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13. ABSTRACT *(Maximum 200 Words)*

The *PTEN* tumor suppressor gene maps to the 10q23 locus, a chromosomal region frequently altered in prostate cancer. The specific aims of this proposal were to 1) develop high quality antibody reagents to PTEN, 2) to identify downstream targets of PTEN and 3) to study PTEN in primary prostate tumors.

We have successfully developed both polyclonal and monoclonal antibodies to PTEN. One antiserum, C54 is a highly specific, sensitive reagent.

Data from our lab has shown that the protein product (PTEN), when reconstituted to *PTEN* -/- cells, induces a cell cycle arrest in G1. This PTEN function requires the ability of PTEN to act as a lipid phosphatase and in so doing functionally antagonize the phosphatidylinositol-3-kinase (PI3K) pathway. In keeping with this notion, a constitutively active form of Akt, a kinase downstream of PI3K, can override a PTEN induced arrest, PTEN can inhibit Akt kinase activity in cells, and *PTEN*-/- tumors have elevated levels of activated Akt. Thus, Akt appears to be a critical downstream target of PTEN.

Finally, we have shown that PTEN is lost from 20% of primary prostate cancers by immunohistochemistry . Furthermore, loss of PTEN is highly associated with high Gleason grade and high tumor stage.

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Introduction

The molecular mechanisms underlying prostate carcinogenesis remain poorly understood. Clues to the molecular alterations that might be of significance in this disease come, in part, from loss-of-heterozygosity (LOH) studies. In particular, LOH of 10q has been reported to occur in prostate cancer with high frequency (30-60%) (1, 2) and two distinct commonly deleted regions have been identified at 10q22-q24 and 10q25 respectively, implying the presence of putative tumor suppressor genes at these loci.

PTEN/MMAC-1/TEP-1 (referred to hereafter as *PTEN*) is a novel tumor suppressor gene that maps to the 10q23.3 interval (3-5). *PTEN* mutations are common in prostate cancer where biallelic loss has been demonstrated (3, 4, 6-13). The aims of this application are 1) to develop antibody reagents that recognize the endogenous PTEN protein 2) to identify cellular downstream targets of PTEN tumor suppression and 3) to determine the extent to which the PTEN protein is lost in primary prostate cancer.

Body

Technical Objectives:

1. The identification, and characterization of the protein product of the *PTEN* tumor suppressor gene.

2. The identification of candidate phosphorylated substrates of the PTEN protein.

3. To determine whether PTEN immunostaining is of prognostic value in patients with early stage prostate cancer.

Aim 1: The identification, and characterization of the protein product of the *PTEN* tumor suppressor gene

Rabbits and mice were immunized with GST-PTEN fusion proteins produced in *e.coli*. Fusion proteins encoding the PTEN C-terminus, the PTEN N-terminus and the full-length protein were used. Anti-sera from rabbits that have been generated include D7 and D8 to the amino terminus, C54 and C55 to the C-terminus and C-56 to the full-length protein. In addition, several monoclonal antibodies have been generated including PC-15, PC-17 and PC-22.

Of these antisera and antibodies C54 is the highest titer antibody. This antibody specifcially recognizes a 58 Kd protein on immunoblots that co-migrates with IVT PTEN (data not shown). Furthermore, immunoprecipitation with C54, PC15, D7 or C56 followed by western blotting with C54 recognizes the same protein. Further, this protein species is absent from cells that are known to have sustained truncating mutations or biallelic deletion of *PTEN* ((14) and data not shown). Thus, this protein is the endogenous PTEN protein .

In the course of our work it became clear that recombinant PTEN can act as a lipid and protein phosphatase. With these antibody reagents we have demonstrated that this is the case of the endogenous protein as well. These antibody reagents are now being used to ask whether PTEN localization is regulated and whether the phosphatase activity of the protein is regulated.

Aim 2: To identify candidate downstream phosphorylated targets of PTEN

PTEN is a protein and lipid phosphatase

The protein product of the *PTEN* gene(PTEN) is a dual-specificity phosphatase. Recombinant PTEN is capable of dephosphorylating both tyrosine and threonine phosphorylated substrates, and in addition can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$), a product of phosphatidylinositol-3-kinase activity (PI3K) (15, 16).

PTEN induces a block in the G1 phase of the cell-cycle

In our experiments, 786-0 renal carcinoma cells, which lack PTEN protein, were transiently co-transfected with a plasmid encoding the cell surface marker CD19, and plasmids encoding PTEN or mutant derivatives. The cell-cycle distribution of the CD19+ cells (marking the transfected cells) was determined by staining with FITC-conjugated anti-CD19 and propidium iodide followed by FACS analysis. We found that wild-type PTEN was capable of inducing a G1 block but tumor derived mutants could not(14). We next compared wild-type PTEN to a number of naturally occurring PTEN mutants in the cell-cycle assay, in protein phosphatase assays (using a phospho-tyrosine substrate) and in assays testing dephosphorylation of H^3-1,3,4,5 inositol tetrakisphosphate(H^3-IP_4). This latter assay reflects the ability of PTEN to dephosphorylate PI3,4,5P_3 in vivo (16). One particularly informative mutant was found, PTEN;G129E. This mutant was identified in the germline *PTEN* gene of two independent Cowden's families (17). In our assays it retained phosphatase activity against a protein substrate, but was incapable of arresting cells in G1 and was incapable of dephosphorylating lipid substrates as measured by dephosphorylation of H^3-IP_4. Thus, the ability of PTEN to induce a cell-cycle block correlated best with its ability to dephosphorylate a lipid substrate.

These data raised the possibility that the regulation of downstream targets of PI3K might be critical for PTEN mediated cell-cycle control. One such downstream effector is the proto-oncogene Akt (18). We first sought to determine whether PTEN could negatively regulate Akt kinase activity. A plasmid encoding T7-epitope tagged Akt was transfected into U2-OS cells with either empty vector or with a plasmid encoding PTEN. PTEN co-transfection led to a dramatic down-regulation of Akt kinase activity as measured by immunoprecipitation of Akt followed by an in vitro kinase assay with an Akt substrate. We next asked whether Akt might be able to override a PTEN induced G1 block. While wild-type Akt had a minimal effect on the PTEN induced G1 block, a myristoylated form of Akt, which no longer requires PI-3,4,5-P_3 for activation, was dramatically better at overcoming a PTEN block. In contrast, kinase-inactive versions of both Akt and Myr-AKt were unable to override PTEN effects. These data suggest that PTEN mediated cell-cycle inhibition depends upon negative regulation of the PI3K/Akt signaling pathway. In keeping with the notion that Akt is critical downstream target of PI3K, tumors that lack PTEN have deregulated Akt activity.

These data link Akt and PTEN in a cell cycle pathway downstream of PI3K. In support of the idea that the PTEN/PI3K/Akt pathway can regulate the cell cycle, the expression of activated Akt or of activated PI3K in a serum-starved cell is sufficient to induce S-phase entry (19). Furthermore, in *PTEN* heterozygous mice there is an increase in proliferating cells in the prostate and thyroid glands (20, 21). In addition, in early PTEN-/- embryos there is widespread, excess cellular proliferation preceding embryonic death (22). Thus, PTEN is necessary *in vivo* role for the appropriate regulation cell-cycle progression and cellular proliferation.

Recent data pertinent to Aim 1 and Aim 2

Mapping the minimal PTEN phosphatase domain.

Protein sequence alignment of PTEN to the VHR dual-specificity phosphatase shows that PTEN residues between 10 to 191 could be sufficient to encode an active phosphatase domain (Fig. 2A and 4A, light gray box). On the other hand numerous tumor-derived mutations are found outside of this domain (see Fig. 4A). We therefore wanted to know what PTEN residues were required for protein and lipid phosphatase activity. In addition, while phosphatase activity appears to be necessary for tumor suppression, whether the minimal PTEN phosphatase domain is sufficient for tumor suppression is not known.

To address this question we have mapped the minimal active phosphatase domain of PTEN. Recombinant GST-PTEN protein and C-terminal truncated forms thereof, were purified from *E. coli* and tested *in vitro* for protein phosphatase activity using ^{32}P-phosphorylated poly(glu4:tyr1) or for lipid phosphatase activity using ^{3}H-1,3,4,5 inositol tetrakisphosphate (^{3}H-IP_4) (Fig 2A-C). In these experiments, the C-terminal boundary of the phosphatase domain was mapped to between residues 344 and 352. Similarly, N-terminal mutations at residues 10 and 35 were tested (Fig. 2A, D, E) and found to be active and inactive respectively thus mapping the N-terminal boundary to between residues 11 and 34. Finally, the minimal domain residues 10-353

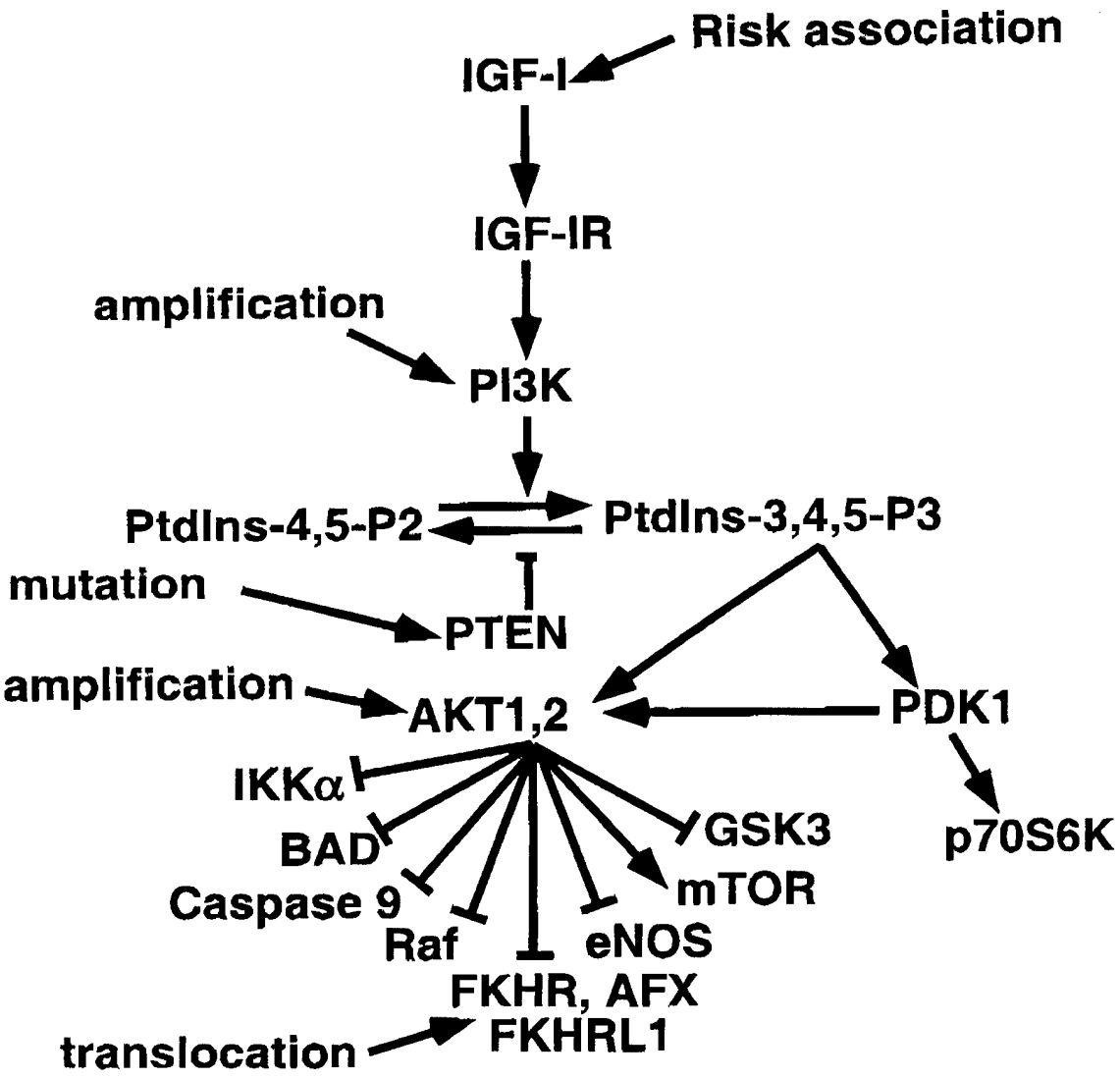
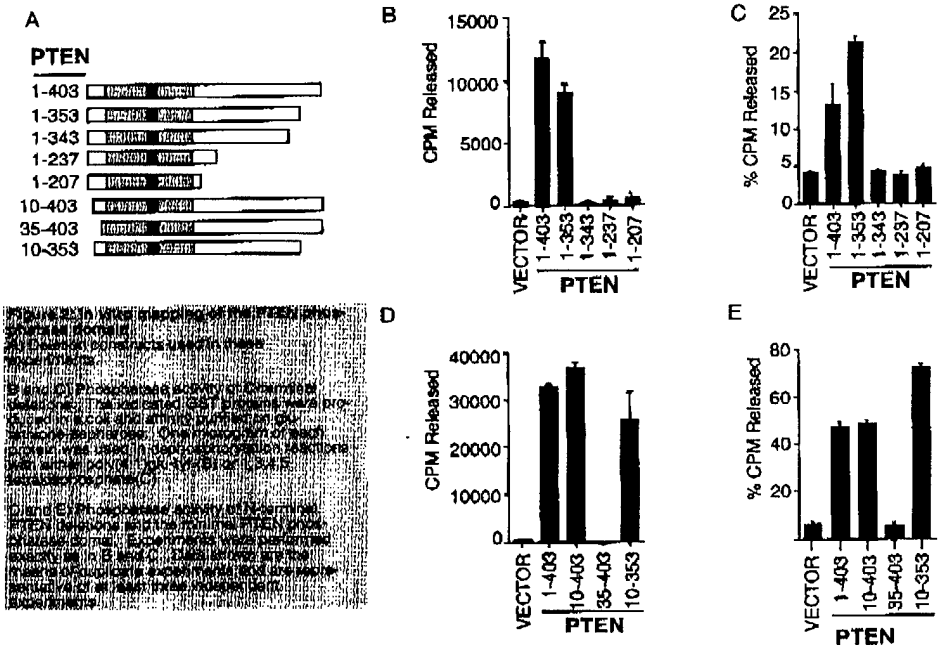


Figure 1: The Phosphoinositide-3 kinase pathway and mechanisms of deregulation in human tumors.

FIGURE 2


A
PTEN
1-403
1-353
1-343
1-237
1-207
10-403
35-403
10-353
B
CPM Released
15000
10000
5000
0
VECTOR
1-403
1-353
1-343
1-237
1-207
PTEN
C
% CPM Released
25
20
15
10
5
0
VECTOR
1-403
1-353
1-343
1-237
1-207
PTEN
D
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40000
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VECTOR
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35-403
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PTEN
E
% CPM Released
80
60
40
20
VECTOR
1-403
10-403
35-403
10-353
PTEN

was tested and found to be active (Fig. 2A, D, E). Thus, this domain is sufficient for both protein and lipid phosphatase activity and in addition, is much larger than would be predicted based upon sequence comparison.

In order to ask whether the minimal phosphatase domain was sufficient to induce a G1 arrest, expression plasmids encoding wild-type or truncated HA-PTEN proteins were introduced into 786-O cells along with an expression plasmid (pCD19) encoding the B-cell surface marker CD19. The cell-cycle profile of the transfected (CD19+) cells was determined by two-color FACS (23). Here, the minimal domain required for enacting a G1 arrest was the same as that required to act as an *in vitro* phosphatase (Fig. 3A, B, D, E). Next, we asked whether the requirements for inhibition of Akt were the same. A plasmid encoding T7-epitope tagged Akt was transfected with vector alone, or with plasmids encoding either PTEN;WT or the indicated mutants. Here, the minimal phosphatase domain (10-353) was sufficient for inhibition of Akt kinase activity (Fig. 3C, F). These data show that the minimal PTEN phosphatase domain is encoded by residues 10-353, and that this domain, when overexpressed, is sufficient to regulate Akt and induce a G1 block.

Returning to the question of how tumor-derived mutations alter PTEN function, it turns out that the majority of PTEN somatic or germ line mutations map to the minimal phosphatase domain that we have defined (Fig.4A, large darker gray box). Furthermore, the mutations that map to this region are universally defective in the *in vitro* phosphatase assays (Fig.4B, C) (14, 15, 24). These mutants also lack Akt and cell cycle inhibitory activity (data not shown).

There are however, a small number of truncation and missense mutations that lie C-terminal to residue 353 (Fig.4A) (25-27). The presence of such mutations raises the possibility that the C-terminal 50 amino acids PTEN might play a role in PTEN tumor suppressor function. Alternatively, deletions or alterations within this region could still impair phosphatase activity by disrupting the overall structure of the protein. To tests this, PTEN proteins truncated at residues 393 and 373 and 377 were generated and tested *in vitro* for inositol phosphatase activity and *in vivo* in the cell cycle assay (Fig. 4D-F and data not shown). Truncations within this region did not alter phosphatase activity nor did they alter the ability of PTEN to inhibited cell cycle progression (Fig. 4D).

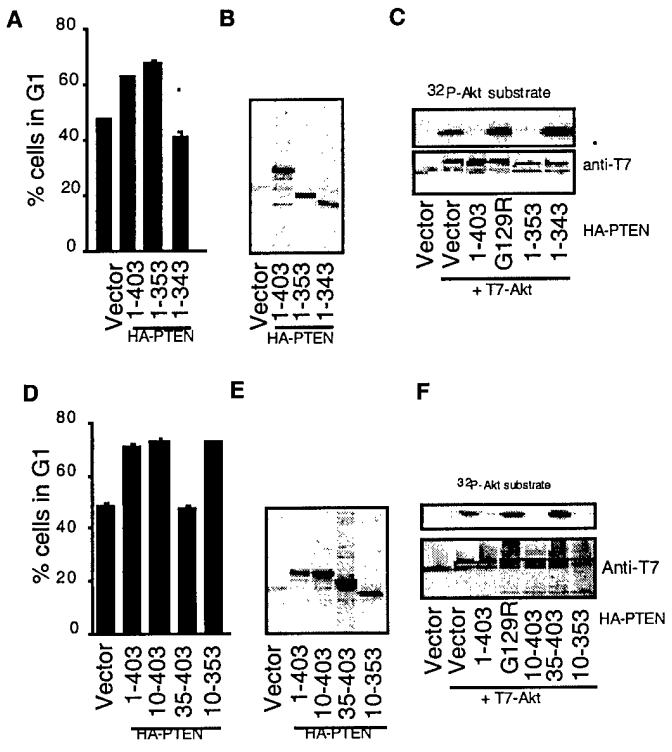

Figure 3: The minimal PTEN phosphatase domain is sufficient for induction of a G1 block and for regulation of Akt kinase activity.
A) Plasmids encoding the indicated C-terminal PTEN truncations mutants were transfected into 786-O cells with pCD19. The cell-cycle profile for the CD19 positive cells was determined by co-staining with propidium iodide and FITC-conjugated anti-CD19.
B) Production of C-terminal PTEN truncation mutants in 786-O cells. 786-O cells were transfected with the indicated plasmids, and metabolically labeled with ^{35}-S methionine. Anti-HA immunoprecipitates were separated by gel electrophoresis and detected by fluorography.
C) Inhibition of Akt kinase activity in cells. Either the vector plasmid or plasmids encoding the indicated C-terminal PTEN truncation mutants were transfected into 786-O cells along with a T7-Akt expression plasmid. Anti-T7 immunoprecipitates were collected on Protein-A sepharose and used for *in vitro* kinase assays with a GST-Akt peptide substrate(as described in Ramswamy et. al., 1999). 32P incorporation into the substrate was determined by autoradiography (upper panel). The production of the T7-Akt protein was assessed by anti-T7 immunoblotting (lower panel).
D) N-terminal and minimal phosphatase domain truncation mutants were scored in the cell-cycle arrest assay as in (A).
E) Production of N-terminal truncation mutants as in (B).
F) Inhibition of Akt kinas activity by N-terminal and the minimal phosphatase domain as in (C).

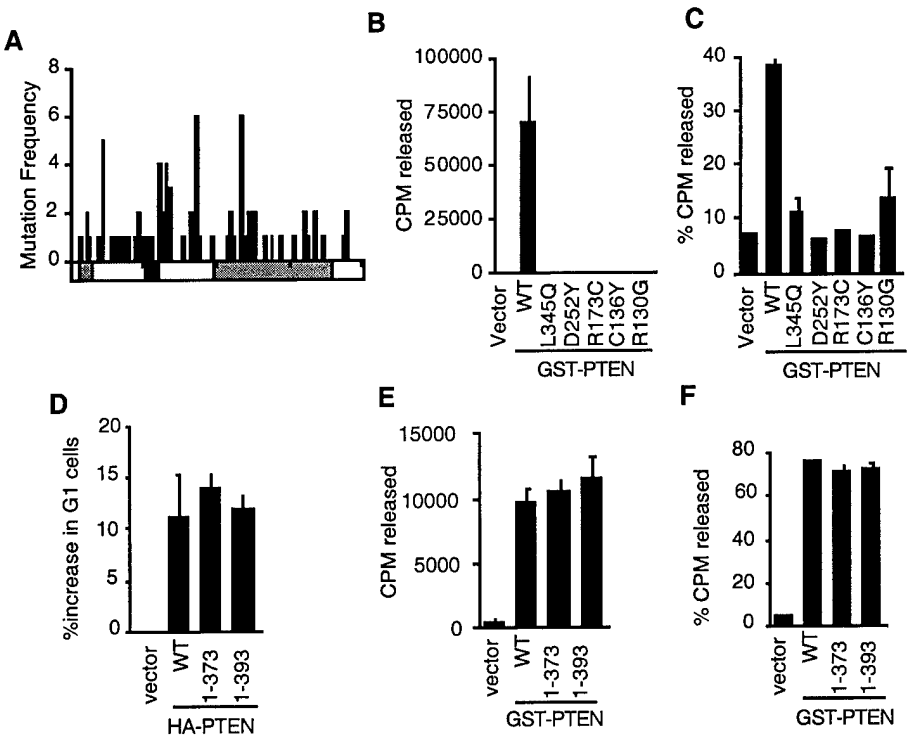

Figure 4: Tumor derived mutations within the minimal PTEN phosphatase domain lead to loss of *in vitro* phosphatase activity, while truncation mutations outside of this domain preserve phosphatase activity and the ability to induce a G1 arrest .
A) A histogram of all published PTEN mutations shown along the coding region of PTEN. The light gray indicates the region of homology to VHR phosphatase. The dark gray region is the minimal active phosphatase domain 10-353. The central dark box represents the catalytic core.
B and C) The indicated GST-PTEN wild-type and mutant proteins were produced in *e.coli*, affinity purifed on glutathione-sepharose and used to dephosphorylate 32P-labeled poly(4:1)glu-tyr (B), or 1,3,4,5 tetrakisphosphate (C).
D) Truncations at resideus 373 and 393 do not interfere with PTEN induction of a cell-cycle block. Plasmids encoding the indicated PTEN proteins were transfected into 786-O cells along with pCD19. Cell-cycle distribution of transfected cells was determined by two-color FACS.
E and F) The indicated GST-PTEN proteins were tested in dephosphorylation assays as in B and C.

These data show that a large number of PTEN mutations inactivate PTEN by altering the phosphatase domain that we have defined (10-353). In addition, these data demonstrate that the 50 C-terminal PTEN residues are not required for the enzymatic activity of PTEN and that mutations in this domain do not affect the function of the phosphatase domain. Thus, how tumor-derived mutations in C-terminus alter PTEN function is not known. We next would like to ask whether the C-terminus is or is not required for tumor suppression and for inhibition of Akt activity when PTEN is expressed at or below endogenous levels

To address this question, we generated amphotrophic retroviruses directing the expression of HA-PTEN;WT and phosphatase inactive mutants. When 786-O renal carcinoma cells are infected with these viruses and selected in puromycin, there is no suppression of growth on plastic dishes (data not shown). These cells express HA-PTEN and the phosphatase inactive mutants at physiologic levels (Fig 5A). However wild-type HA-PTEN, but not HA-PTEN mutant infected cells do show a serum withdrawal-induced G1 arrest and markedly reduced colony formation in soft-agar (Fig.5C and data not shown).

PTEN phosphorylation

PTEN participates in a signal transduction pathway consisting of receptor, membrane bound and cytosolic kinases. In this context we asked whether PTEN is a phosphoprotein itself and whether phosphorylation of PTEN impacts upon PTEN function.

PTEN is a phosphoprotein.

To test for *in vivo* PTEN phosphorylation, C54 (anti-PTEN) and preimmune immunoprecipitates were prepared from ^{32}P-orthophosphate labeled ACHN cells containing wild-

type PTEN. Bound proteins were separated by gel electrophoresis and detected by autoradiography. C54 IP's contain a ^{32}P labeled (Fig 6A) that immunoblots with C54 anti-sera (data not shown). Similarly, transfected HA-tagged PTEN incorporates ^{32}P-orthophosphate during metabolic labeling (Fig 6B). Phosphoamino acid analysis of the phosphorylated endogenous protein demonstrated that predominant phosphoamino acids are serine and threonine (data not shown).

PTEN phosphorylation maps to the C-terminus. In order to understand the functional consequence of PTEN phosphorylation we have decided to first localize phosphorylation sites and then study the consequence of mutation of these sites. To this end, ^{32}P-labeled HA-PTEN isolated from transfected U2-OS cells was subjected to CNBr cleavage. The resulting peptides were separated on a tricine gradient gel where a single CNBr cleavage product was seen that based upon size is predicted to encompass PTEN residues 270-403 (Fig. 6C). This led us to test our series of C-terminal PTEN truncation mutants for phosphorylation by transfection and *in vivo* labeling with ^{32}P. Phosphorylation of PTEN was eliminated by deletion at residue 353 (Fig. 7A). The C-terminus of PTEN contains two potential tryptic peptides (Fig. 7B). When GST-PTEN is digested with trypsin the unphosphorylated forms of these two peptides can be detected by MALDI-TOF (matrix-assisted laser-desorption ionization time-of-flight) mass spectroscopy (Fig. 7C). However, the phosphorylated forms of these peptides have, to date not resolved in 2D peptide mapping experiments (data not shown). Thus, while the C-terminus is required for phosphorylation of PTEN, the exact sites of *in vivo* phosphorylation are not yet known.

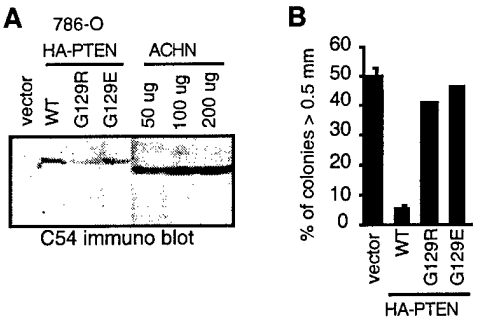

Figure 5: Infection of 786-O cells with retrovirus encoding wild-type PTEN, but not PTEN mutants inhibits growth in soft-agar.
A) Production of the indicated HA-PTEN proteins after retroviral infection compared to endogenous PTEN in ACHN renal carcinoma cells. 50 ug of 786-0 cells infected with the indicated retrovirus was immunoblotted with anti-PTEN C54 antibodies. The lanes shown are from the same exposure of the same blot and were reordered for clarity.
B) Infected 786-O cells were grown suspended in soft agar for 10 days. At the end of 10 days 100 colonies were counted. Shown is the percentage of colonies greater than 0.5 arbitrary scale units at 100X magnification.

Within the first C-terminal tryptic peptide (residues 348-378) (3145 daltons) there is a GSK3 consensus phosphorylation site. As GSK3 is a component of the PI3K/Akt cascade (Fig 1), we asked whether GSK3ß could phosphorylate PTEN *in vitro*. GST-PTEN and truncation mutants thereof were produced in *E. coli* and used as substrates for purified GSK3ß. GSK3 phosphorylated PTEN and mapping showed a requirement for the C-terminus (Fig. 8A). In parallel experiments, Akt and PI3K did not induce PTEN phosphorylation (data not shown). To locate the site of phosphorylation by GSK-3ß, GST-PTEN phosphorylated by GSK3ß was digested with trypsin and tryptic peptides were separated by HPLC. One phosphorylated peptide was found in fraction 32 (Fig. 8B). The mass of this peptide is 3145 daltons as measured by MALDI-TOF. This is consistent with peptide 348-378 (Fig. 7B and data not shown). This phosphorylated peptide was subjected Edman degradation sequencing (Fig. 8D). The liberated counts were primarily localized to serine 362. In addition, some counts were obtained at threonine 367. Residues S362 and T367 do form a GSK-3ß consensus sequence. Finally, phosphoamino acid analysis of this peptide revealed the presence of both phosphoserine and to a lesser extent phosphothreonine (Fig. 8C). Thus, PTEN is an *in vitro* substrate for GSK3ß. Whether this is true *in vivo* and how GSK-3 phosphorylation might alter PTEN function will be addressed in future experiments.

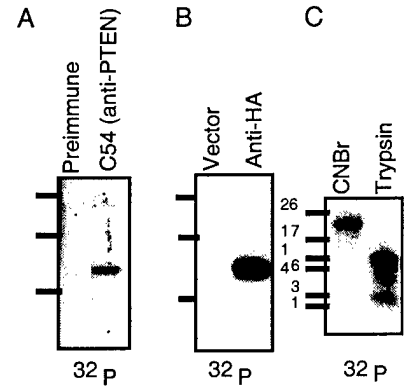

Figure 6: PTEN is a phosphoprotein.
A) C54 immune and preimmune IP's were prepared from U2-OS cells labeled with ^{32}P-orthophosphate. Bound proteins were separated by electrophoresis and detected by autoradiography.
B) U2-OS cells, transfected with vector or an HA-PTEN;WT plasmid were labeled with ^{32}P-orthophosphate. Anti-HA IP's were processed as in A.
C) CNbr and tryptic cleavage of the PTEN band seen in B. Peptides were resolved on a 16% Tris-tricine gel and detected by autoradiography.

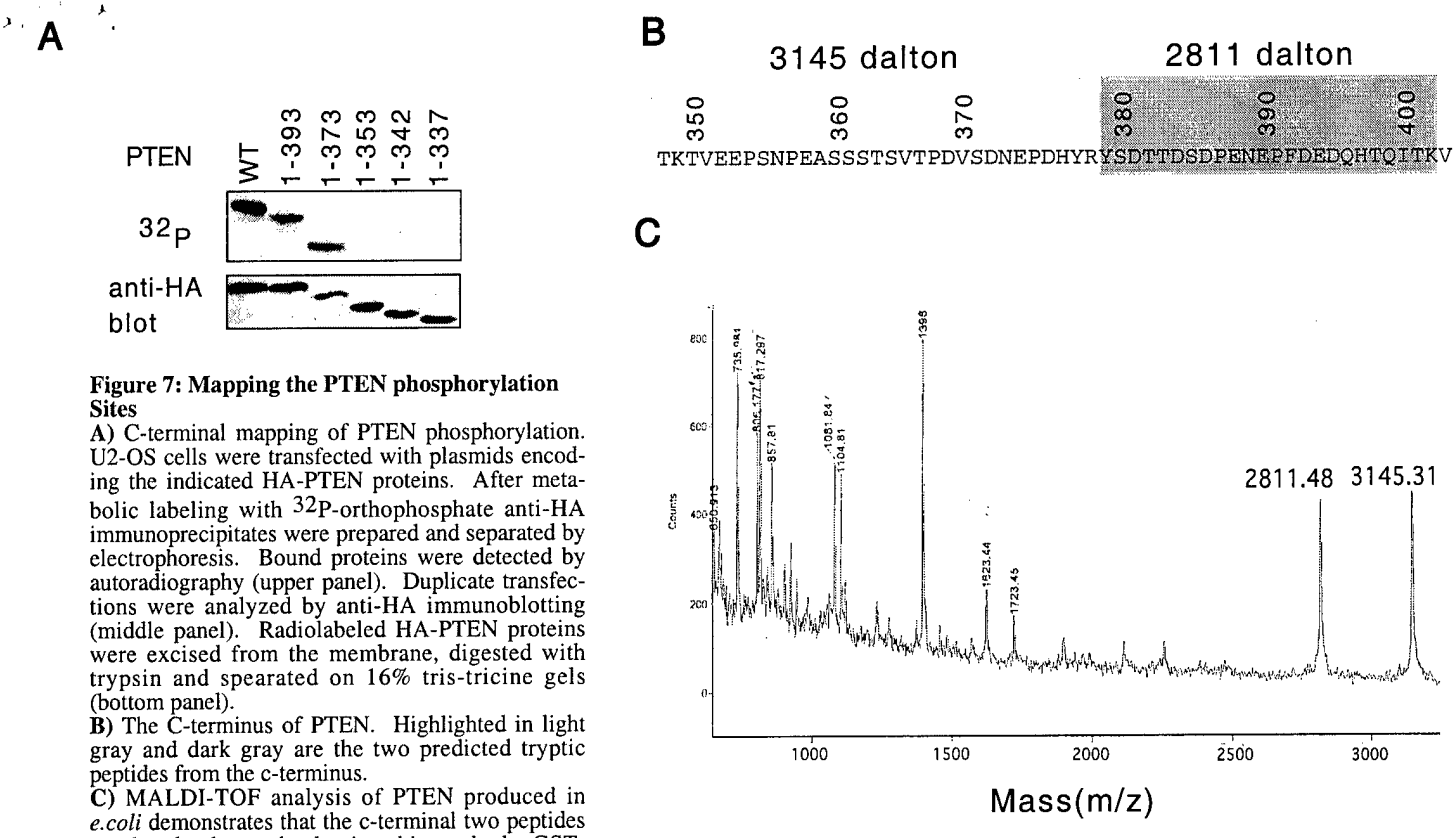

Figure 7: Mapping the PTEN phosphorylation Sites

A) C-terminal mapping of PTEN phosphorylation. U2-OS cells were transfected with plasmids encoding the indicated HA-PTEN proteins. After metabolic labeling with ^{32}P-orthophosphate anti-HA immunoprecipitates were prepared and separated by electrophoresis. Bound proteins were detected by autoradiography (upper panel). Duplicate transfections were analyzed by anti-HA immunoblotting (middle panel). Radiolabeled HA-PTEN proteins were excised from the membrane, digested with trypsin and spearated on 16% tris-tricine gels (bottom panel).

B) The C-terminus of PTEN. Highlighted in light gray and dark gray are the two predicted tryptic peptides from the c-terminus.

C) MALDI-TOF analysis of PTEN produced in *e.coli* demonstrates that the c-terminal two peptides can be clearly resolved using this method. GST-PTEN was produced in e.coli, affinity purifed on glutathione-sepharose, cleaved with thrombin, purified on a benzamidine column, and then concentrated on a desalting column. 500 ng of protein was digested in-gel and extracted for MALDI-TOF analysis.

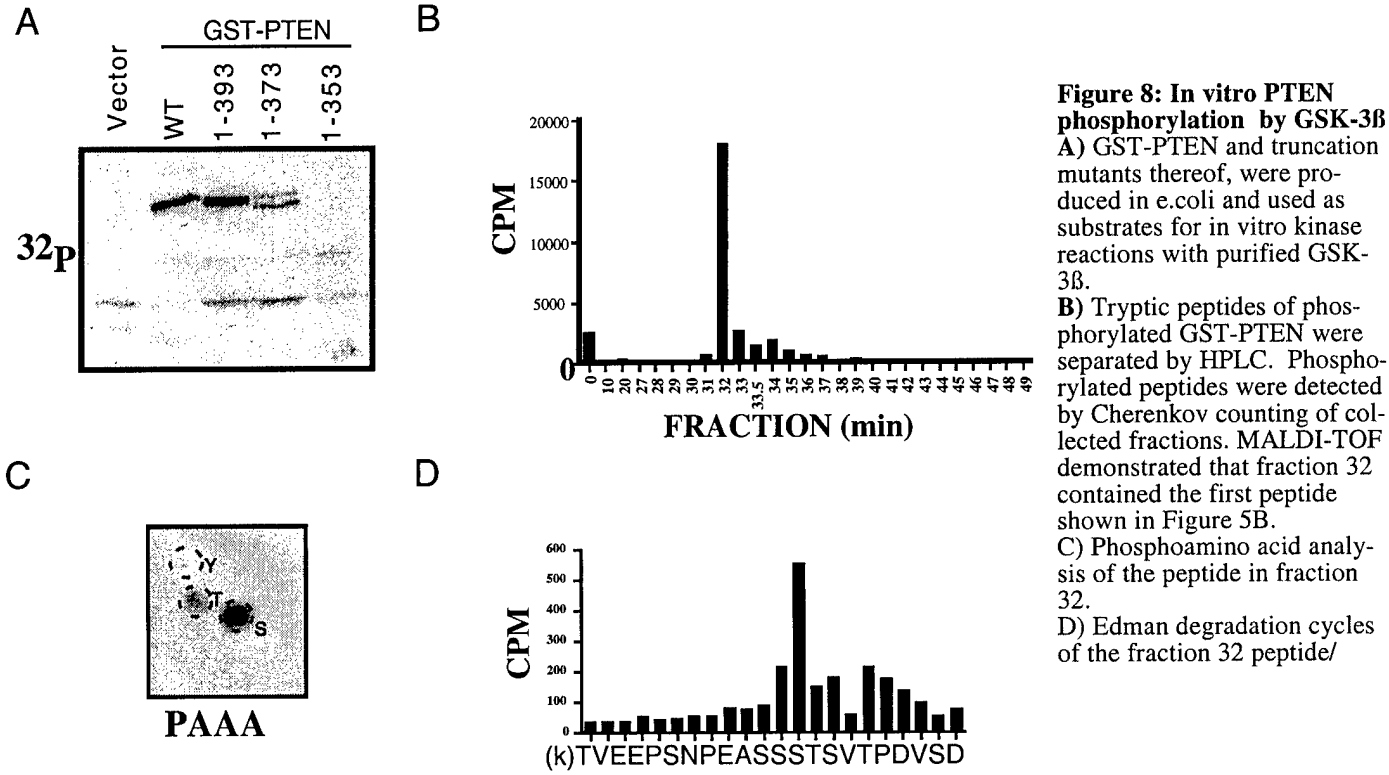

Figure 8: In vitro PTEN phosphorylation by GSK-3ß

A) GST-PTEN and truncation mutants thereof, were produced in e.coli and used as substrates for in vitro kinase reactions with purified GSK-3ß.

B) Tryptic peptides of phosphorylated GST-PTEN were separated by HPLC. Phosphorylated peptides were detected by Cherenkov counting of collected fractions. MALDI-TOF demonstrated that fraction 32 contained the first peptide shown in Figure 5B.

C) Phosphoamino acid analysis of the peptide in fraction 32.

D) Edman degradation cycles of the fraction 32 peptide/

Aim 3. To determine whether PTEN immunostaining is of prognostic value in patients with early stage prostate cancer.

A murine polyclonal antiserum was raised against a glutathione-S-transferase fusion polypeptide of the carboxyl terminus of PTEN. Archival paraffin tissue sections from 109 cases of resected prostate cancer were immunostained with the antiserum, utilizing DU145 and PC-3 cells as positive and negative controls respectively. PTEN expression was seen in the secretory cells. Cases were considered positive when granular cytoplasmic staining was seen in all tumor cells; mixed, when areas of both positive and negative tumor cell clones were seen; and negative when no tumor but adjacent benign prostate tissue showed positive staining. Seventeen cases (15.6%) of prostate cancer were positive, 70 (64.2%) were mixed and 22 (20.2%) were negative. Total absence of PTEN expression correlated with Gleason score ($p=0.0081$), correlated more significantly with a Gleason score of 7 or higher ($p=0.0004$) and with advanced pathological stage (American Joint Committee on Cancer (AJCC) T3c, T4) ($p=0.0078$). Thus, loss of PTEN protein is correlated with pathological markers of poor prognosis in prostate cancer (28).

Discussion

To date, our data have demonstrated that the protein product of the PTEN tumor suppressor gene acts as an inhibitor of progression through the G1 phase of the cell cycle. This PTEN function requires the ability to antagonize the PI3K/Akt pathway via PTEN's lipid phosphatase activity. Further, we have mapped the minimal phosphatase domain and showed that this domain is sufficient to induce a G1 arrest. This domain is frequently targeted by somatic and germline PTEN mutations.

Our data in human prostate tumors has demonstrated that PTEN loss is highly correlated with increased Gleason score and with advanced tumor stage and thus is likely to be associated with a poorer prognosis.

Our current studies are focused on defining the components of the Akt pathway that lie downstream of tumor suppression by PTEN. These data from these experiments is directed at trying to determine whether Akt is an appropriate target for new small molecule inhibitor development. In addition, we are currently generating mouse prostate tumor models based upon forced overexpression of Akt and PI3K in the prostate.

Key Research Accomplishments

1. Identified cell cycle regulation as a key PTEN function.
2. Identified the Pi3K/Akt pathway as a critical downstream target of PTEN action with respect to the cell cycle function.
3. Defined the minimal PTEN phosphatase domain and have shown that this domain is sufficient for inhibition of Akt and for inhibition of cell cycle progression.
4. Showed that loss of PTEN protein in primary prostate tumors is associated with high Gleason score and advanced tumor stage.
5. Developed high quality anti-PTEN antisera and identified the endogenous PTEN protein.

Reportable Outcomes

1. 1. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., **Sellers, W. R.** Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway, Proc Natl Acad Sci U S A. *96:* 2110-2115, 1999.

2. 1. McMenamen, M., Lee, P., Perera, S., Kaplan, I., Loda, M., and **Sellers, W. R.** Loss of staining of the Tumor suppressor protein PTEN in primary prostate cancer correlates with Gleason grade, Cancer Res. *(in press):*, 1999.

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Conclusions

Our work has shown that PTEN is an important regulator of prostate carcinogenesis. In this regard PTEN loss in the prostate appears to have prognostic import though definitive studies using larger tumor repositories are underway. The discovery of the PI3K/Akt pathway as a critical downstream target of PTEN inhibitory function leads one to believe that the development of small molecule inhibitors of this pathway might be of therapeutic value in the treatment of prostate cancer. Mouse models are being developed to try and validate Akt as a target for drug therapy.

References

1. Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J., and Spurr, N. K. Loss of the chromosomal region 10q23-25 in prostate cancer, Cancer Res. *55:* 4800-3, 1995.

2. Komiya, A., Suzuki, H., Ueda, T., Yatani, R., Emi, M., Ito, H., et al. Allelic losses at loci on chromosome 10 are associated with metastasis and progression of human prostate cancer, Genes Chrom Canc. *17:* 245-53, 1996.

3. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, Nat Genet. *15:* 356-62, 1997.

4. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, Science. *275:* 1943-7, 1997.

5. Li, D. M. and Sun, H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta, Cancer Res. *57:* 2124-9, 1997.

6. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., et al. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression, Proc Natl Acad Sci U S A. *95:* 5246-50, 1998.

7. Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines, Cancer Res. *57:* 5221-5, 1997.

8. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer, Cancer Res. *57:* 4997-5000, 1997.

9. Wang, S. I., Parsons, R., and Ittmann, M. Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas, Clin Cancer Res. *4:* 811-5, 1998.

10. Pesche, S., Latil, A., Muzeau, F., Cussenot, O., Fournier, G., Longy, M., et al. PTEN/MMAC1/TEP1 involvement in primary prostate cancers, Oncogene. *16:* 2879-83, 1998.

11. Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., et al. Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues, Cancer Res. *58:* 204-9, 1998.

12. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts, Cancer Res. *58:* 2720-3, 1998.

13. Gray, I. C., Stewart, L. M., Phillips, S. M., Hamilton, J. A., Gray, N. E., Watson, G. J., et al. Mutation and expression analysis of the putative prostate tumour- suppressor gene PTEN, Br J Cancer. *78:* 1296-300, 1998.

14. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., et al. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway, Proc Natl Acad Sci U S A. *96:* 2110-2115, 1999.

15. Myers, M. P., Stolarov, J., End, C., Li, J., Wang, S. I., Wigler, M. H., et al. P-TEN, the tumor supressor from human chromosme 10q23 is a dual-specificity phosphatase, Proc. Natl. Acad. Sci USA. *94:* 9052-9057, 1997.

16. Maehama, T. and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, Phosphatidylinositol 3,4,5-Triphosphate, J Biol Chem. *273:* 13375-13378, 1998.

17. Marsh, D. J., Coulon, V., Lunetta, K. L., Rocca-Serra, P., Dahia, P. L., Zheng, Z., et al. Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation, Human Molecular Genetics. *7:* 507-15, 1998.

18. Downward, J. Mechanisms and consequences of activation of protein kinase B/Akt, Curr Opin Cell Biol. *10:* 262-7, 1998.

19. Klippel, A., Escobedo, M. A., Wachowicz, M. S., Apell, G., Brown, T. W., Giedlin, M. A., et al. Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation [In Process Citation], Mol Cell Biol. *18:* 5699-711, 1998.

20. Di Cristafano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. PTEN is essential for embryonic development and tumor suppression, Nat Genet. *19:* 348-355, 1998.

21. Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., et al. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems [In Process Citation], Proc Natl Acad Sci U S A. *96:* 1563-8, 1999.

22. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, Cell. *95:* 29-39, 1998.

23. Sellers, W. R., Novitch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., et al. Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth, Genes & Development. *12:* 95-106, 1998.

24. Furnari, F. B., Huang, H. J., and Cavenee, W. K. The phosphoinositol phosphatase activity of PTEN mediates a serum- sensitive G1 growth arrest in glioma cells, Cancer Res. *58:* 5002-8, 1998.

25. Kong, D., Suzuki, A., Zou, T. T., Sakurada, A., Kemp, L. W., Wakatsuki, S., et al. PTEN1 is frequently mutated in primary endometrial carcinomas [letter], Nature Genetics. *17:* 143-4, 1997.

26. Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., et al. PTEN gene mutations are seen in high-grade but not in low-grade gliomas, Cancer Res. *57:* 4187-90, 1997.

27. Rhei, E., Kang, L., Bogomolniy, F., Federici, M. G., Borgen, P. I., and Boyd, J. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas, Cancer Res. *57:* 3657-9, 1997.

28. McMenamen, M., Lee, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. Loss of staining of the Tumor suppressor protein PTEN in primary prostate cancer correlates with Gleason grade, Cancer Res. *(in press):*, 1999.

Appendices

1. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., et al. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway, Proc Natl Acad Sci U S A. *96:* 2110-2115, 1999

2. McMenamen, M., Lee, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. Loss of staining of the Tumor suppressor protein PTEN in primary prostate cancer correlates with Gleason grade, Cancer Res (in press), 1999.

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Cell Biology

Regulation of G_1 progression by the *PTEN* tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway

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ABSTRACT ***PTEN/MMAC1* is a tumor suppressor gene located on chromosome 10q23. Inherited *PTEN/MMAC1* mutations are associated with a cancer predisposition syndrome known as Cowden's disease. Somatic mutation of PTEN has been found in a number of malignancies, including glioblastoma, melanoma, and carcinoma of the prostate and endometrium. The protein product (PTEN) encodes a dual-specificity protein phosphatase and in addition can dephosphorylate certain lipid substrates. Herein, we show that PTEN protein induces a G_1 block when reconstituted in *PTEN*-null cells. A *PTEN* mutant associated with Cowden's disease (PTEN;G129E) has protein phosphatase activity yet is defective in dephosphorylating inositol 1,3,4,5-tetrakisphosphate *in vitro* and fails to arrest cells in G_1. These data suggest a link between induction of a cell-cycle block by PTEN and its ability to dephosphorylate, *in vivo,* phosphatidylinositol 3,4,5-trisphosphate. In keeping with this notion, PTEN can inhibit the phosphatidylinositol 3,4,5-trisphosphate-dependent Akt kinase, a downstream target of phosphatidylinositol 3-kinase, and constitutively active, but not wild-type, Akt overrides a PTEN G_1 arrest. Finally, tumor cells lacking PTEN contain high levels of activated Akt, suggesting that PTEN is necessary for the appropriate regulation of the phosphatidylinositol 3-kinase/Akt pathway.**

Abnormalities of chromosomal region 10q23 are frequent in a number of malignancies, including prostate cancer and glioblastoma (1, 2). Recently, a candidate tumor suppressor gene *PTEN/MMAC1/TEP1* (for simplicity hereafter referred to as *PTEN*) was cloned and mapped to this region (3–5). Somatic mutations of *PTEN* are found in a number of malignancies, including glioblastoma, melanoma, and carcinomas of the prostate, lung, endometrium, and head and neck (3, 4, 6–14). Germ-line mutations of the *PTEN* gene are associated with the development of Cowden's disease (CD) and Bannayan–Zonana syndrome (BZS) (15–18). CD is characterized by the occurrence of multiple hamartomas in the skin, gastrointestinal tract, breast, thyroid, and central nervous system and an increased incidence of breast and thyroid cancers (18). BZS is a related syndrome in which intestinal hamartomas are accompanied by neurological abnormalities including mild mental retardation, delayed motor development, vascular malformations, and speckled penis (18).

The predicted protein product of the *PTEN* gene (referred to hereafter as PTEN) has homology to tensin, an actin binding protein localized to focal adhesion complexes (19); to auxilin, a protein involved in the uncoating of clatherin-coated vesicles (20); and to dual-specificity phosphatases (4, 21). Recombinant PTEN is capable of dephosphorylating both tyrosine- and threonine-phosphorylated substrates and in addition can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3) (22, 23). Overproduction of PTEN can suppress colony formation in certain cells, growth in soft agar, and tumor formation in nude mice (24, 25). Recent data suggest that PTEN might function, at least in part, through regulation of focal adhesion kinase and the subsequent inhibition of adhesion and migration (26). PTEN is essential for murine embryonic development beyond day 7.5. In the mouse loss of *PTEN* allele leads to hyperplasia and dysplasia in the skin, gastrointestinal tract, and prostate, as well as tumor formation (27).

In this study, we found that reintroduction of a PTEN cDNA into cells lacking a wild-type PTEN protein led to a cell-cycle block in G_1. This function was tightly linked to the phosphatase activity of PTEN and was inactivated by tumor-derived mutations. Furthermore, a PTEN mutant, associated with CD, that retains protein phosphatase activity was defective in arresting cells in G_1 and was also defective in dephosphorylating inositol 1,3,4,5-tetrakisphosphate (IP_4).

These data suggested that PTEN might regulate cell-cycle progression by blocking activation of downstream targets of phosphatidylinositol 3-kinase such as the protooncogene Akt. In keeping with this notion, PTEN was capable of inhibiting wild-type Akt kinase activity in cells. Furthermore, a constitutively active form of Akt, but not wild-type Akt, overrode a PTEN-induced cell-cycle block.

MATERIALS AND METHODS

Cell Culture, Transfection, and Metabolic Labeling. ACHN, 786-O, SAOS-2, and U2-OS cells (gifts from the Kaelin laboratory) were maintained in DMEM containing 10% Fetal Clone (HyClone), penicillin and streptomycin at 37°C. Cells were transfected with Fugene 6 (Boehringer-Mannheim) for 786-O cells or by the calcium phosphate procedure for U2-OS, ACHN, and SAOS-2 cells, as described (28, 29). Transfected 786-O cells were metabolically labeled for 3 h in 5 ml of methionine-free medium supplemented with 10% dialyzed fetal calf serum and [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq).

Plasmids. A cDNA fragment encoding PTEN amino acid residues 1–403 was PCR-amplified from a 293 cDNA library (30) and ligated to vector pSG5L-HA (28) to give pSG5L-HA-PTEN;WT. An Akt-1 cDNA was amplified by reverse transcription-coupled PCR from total HeLa cell RNA and reamplified with a 5′ primer containing a Kozak sequence and

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Abbreviations: IP_4, inositol 1,3,4,5-tetrakisphosphate; PtdIns, phosphatidylinositol 3,4,5-trisphosphate; CD, Cowden's disease; HA, hemagglutinin; GST, glutathione *S*-transferase.

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sequences encoding a hemagglutinin (HA) epitope and cloned into pLNCX to give pLNCX-HA-Akt. A double-stranded oligonucleotide encoding the src myristoylation sequence was inserted 5′ of the HA tag to generate pLNCX-Myr-HA-Akt. pSG5L-HA-PTEN;C124S, pSG5L-HA-PTEN;G129R, pSG5L-HA-PTEN;G129E, pSG5L-HA-PTEN;1–274, pSG5L-HA-PTEN;1–336 and pSG5L-HA-PTEN;Δ274–342, pLNCX-HA-Akt;K179M, pLNCX-myr-HA-Akt;K179M were generated by site-directed mutagenesis or by PCR mutagenesis. Inserts from the pSG5L-HA-PTEN plasmids were cloned into pGEX2T to give the corresponding pGEX2T-PTEN plasmids. A cDNA for AKT-1 was PCR-amplified from a fetal brain library and ligated to the vector from pCDNA3-T7-VHL to give pCDNA3-T7-AKT. All plasmid inserts obtained by PCR or altered by site-directed mutagenesis were verified by sequencing. pCD19 has been previously described (31).

Antibodies. Production of PTEN antiserum (C54) will be described elsewhere (32). HA.11, fluorescein isothiocyanate-conjugated anti-CD19, anti-T7, and anti-phospho-Akt (Ser-473) antibodies were obtained from Babco (Richmond, CA), Novagen, Caltag (South San Francisco, CA), and New England Biolabs, respectively.

Immunoprecipitations and Immunoblotting. Preparation of whole cell extracts, immunoprecipitations, and immunoblotting conditions are as described (28). For immunoblotting, C54 antiserum was diluted 1:500 in TBS/4% milk. Secondary antibodies, alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit (Southern Biotechnology Associates) were diluted 1:5,000. For chemiluminescent detection, horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology) was used at a 1:2,000 dilution and detected with the SuperSignal kit (Pierce).

Fluorescence-Activated Cell Sorting. Cells grown on p100 plates were transfected with 4 μg of pCD19 and either 11 μg (Fugene 6 transfections) or 21 μg (calcium phosphate transfections) of the backbone pSG5L plasmid or pSG5L plasmids encoding PTEN or the indicated PTEN mutants. Cell-cycle determination of the CD19+ cells was carried out as described (28).

Protein and Inositol Phosphatase Assays. Poly(Glu_4-Tyr_1) copolymer (Sigma) was phosphorylated *in vitro* essentially as described (22). Briefly, poly(Glu_4-Tyr_1) was resuspended at a final concentration of 3.3 mg/ml in 50 mM Tris·HCl (pH 7.4), 2 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.1 mM ATP in a reaction mixture containing 10 μCi of [γ-^{33}P]ATP and 100 units of β-insulin receptor kinase (Stratagene) and incubated at 30°C for 4 h. Labeled copolymer was precipitated with 100% trichloroacetic acid (TCA) washed with 20% TCA and acetone, lyophilized, and resuspended in, and dialyzed against 50 mM imidazole (pH 7.2). Protein phosphatase assays were done as described (22). Dephosphorylation of [^{3}H]inositol 1,3,4,5-tetrakisphosphate (NEN) was performed as described by using 1 μg of the relevant glutathione *S*-transferase (GST) fusion proteins (23).

Akt Kinase Assays. U2-OS cells were transfected with plasmids encoding T7-Akt-1 and pSG5L, pSG5L-HA-PTEN, or mutant derivatives. Thirty-six hours after transfection T7 immunoprecipitates were prepared from cell lysates, collected on protein A-Sepharose and incubated in a reaction mixture containing 30 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 20 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 5 μg of a GST fusion protein containing an Akt peptide substrate, for 30 min at 25°C. Radiolabeled substrate was separated from unincorporated [γ-^{32}P]ATP by gel electrophoresis and detected by autoradiography.

RESULTS

PTEN Induces a Block in the G_1 Phase of the Cell Cycle. Attempts at stable expression of PTEN in PTEN-null 786-O renal carcinoma and A172 glioblastoma cell lines failed to yield clonal lines that produced detectable HA-PTEN (data not shown). Next, transient assays were used to determine whether PTEN might be capable of altering cell-cycle progression. 786-O renal carcinoma cells, which lack PTEN protein (Fig. 1*D*), were transiently transfected with either empty vector or plasmids encoding either HA-tagged PTEN (PTEN;WT) or a tumor-derived PTEN catalytic domain mutant (PTEN;G129R) (4), along with a plasmid encoding the cell surface marker CD19 (pCD19). After 40 h, the DNA content of the successfully transfected cells was determined by staining cells with fluorescein isothiocyanate-conjugated anti-CD19 and propidium iodide followed by fluorescence-activated cell sorting. Wild-type PTEN reproducibly induced an increase in the percentage of cells in G_1 when compared with the vector alone or to PTEN;G129R (Fig. 1*A*). In contrast, reintroduction of plasmids encoding either the tumor suppressor proteins pRB or VHL [which is defective in 786-O cells (30)] or the dual-specificity phosphatase cdc25C failed to induce a G_1 arrest in these cells (Fig. 1*A* and data not shown). Production of HA-PTEN in two cell lines that retain endogenous PTEN protein (SAOS-2 and ACHN) did not alter the cell-cycle distribution of these cells (Fig. 1 *B*–D), but, as a positive control in pRB-null SAOS-2 cells, reintroduction of a plasmid encoding pRB did effect a G_1 arrest (Fig. 1*B*). Under these same experimental conditions, production of PTEN protein in 786-O cells did not lead to an increase in the percentage of cells harboring a sub-2*N* DNA content, suggesting that PTEN did not induce apoptosis in these cells (Table 1). Thus, PTEN specifically induced a G_1 block in 786-O cells, which lack PTEN.

Tumor-Derived Mutants Inactivate PTEN Phosphatase Activity and Cell-Cycle Control. A number of tumor-derived PTEN mutations have been reported that lie outside of the predicted phosphatase and tensin–auxilin homology domains. Three such tumor-derived mutants, PTEN;1–274, PTEN;1–336, and PTEN;Δ274–342 were tested and were defective in the cell-cycle assay (Fig. 2*A*). With the exception of PTEN;1–

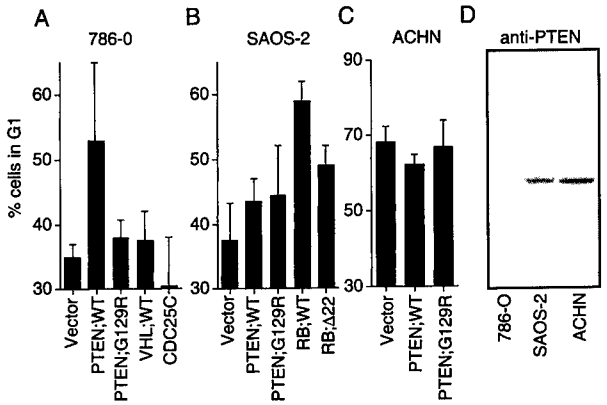

FIG. 1. PTEN induces a G_1 block. (*A*) PTEN, but not pVHL or cdc25C, induced a G_1 block in 786-O cells. 786-O cells were transiently cotransfected with a plasmid encoding CD19 (pCD19) along with plasmids encoding the indicated proteins. Forty hours after transfection, cells were fixed and the cell-cycle distribution of the successfully transfected cells was determined by fluorescence-activated cell sorting analysis. The mean and SEM of two experiments are shown. (*B*) PTEN does not alter the cell-cycle profile of SAOS-2(RB−/−) cells. SAOS-2 cells were transiently transfected with pCD19 and the plasmids encoding the indicated proteins and analyzed as in Fig. 2*A*. The mean and SEM of two experiments are shown. (*C*) PTEN does not alter the cell-cycle profile of ACHN cells. ACHN cells were transiently transfected with pCD19 or plasmids encoding the indicated proteins and analyzed as in *A*. The mean and SEM of two experiments are shown. (*D*) Immunoblot detection of PTEN protein in 786-O, SAOS-2, and ACHN cells. C54 anti-PTEN antiserum was used to detect PTEN by immunoblot analysis of protein extracts from the indicated cell lines.

Table 1. Percentage of CD19+ 786-O cells with sub-2*N* DNA content

Exp.	Vector	PTEN;WT	PTEN;G129R
1	4.6	5.1	5.8
2	2.4	1.5	1.4
3	1.0	1.3	1.0

786-O cells were transiently transfected with pCD19 and the indicated pSGL-HA expression plasmids. After 36 h, cells were harvested and processed as in Fig. 1*A*.

274, these mutant proteins were produced to levels similar to that of wild-type PTEN in 786-O cells (Fig. 2*B*). Two biochemical properties have been ascribed to PTEN. PTEN can dephosphorylate certain protein substrates containing either phosphotyrosine or phosphothreonine (33). In addition, PTEN can dephosphorylate PtdIns-3,4,5-P_3 (23). We next asked whether the three tumor-derived mutants were defective for either of these functions. When produced as GST fusion proteins, all three mutant proteins were defective in catalyzing the release of phosphate from either a ^{33}P-labeled poly(Glu_4-Tyr_1) substrate or [^{3}H]inositol 1,3,4,5-tetrakisphosphate ([^{3}H]IP_4) (Fig. 2 *C* and *D*).

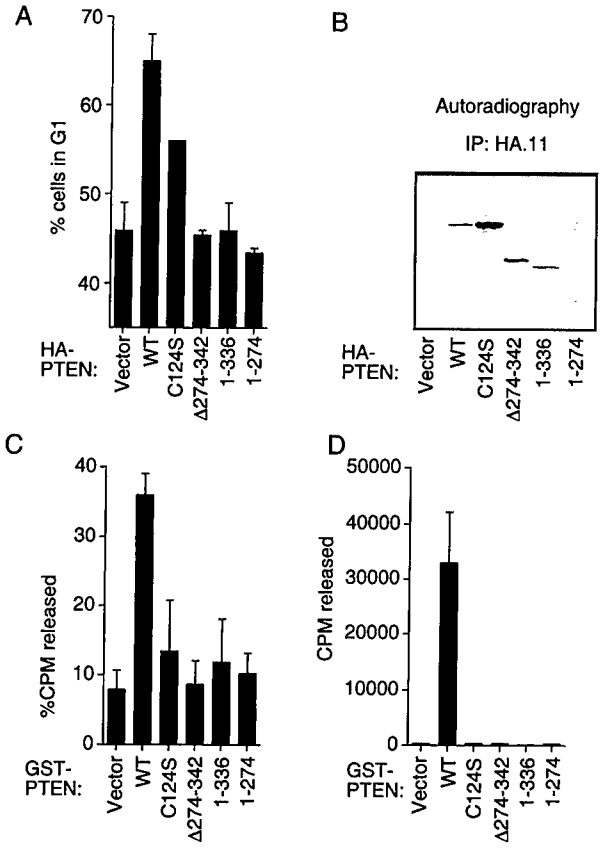

FIG. 2. Substrate trapping variants of PTEN induce a G_1 block but tumor-derived mutants do not. (*A*) Comparison of wild-type, substrate-trapping and C-terminal mutant forms of PTEN in the cell-cycle assay. 786-O cells were cotransfected with pCD19 and plasmids encoding the indicated proteins and analyzed as in Fig. 1*A*. (*B*) Expression of PTEN proteins in 786-O cells. 786-O cells were transfected with plasmids encoding the indicated proteins. Forty hours after transfection anti-HA immunoprecipitates of protein extracts prepared from metabolically labeled cells were separated by gel electrophoresis and subjected to fluorography. (*C*) Inositol phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were used to dephosphorylate [^{3}H]IP_4. (*D*) Protein tyrosine phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were used to dephosphorylate ^{33}P-labeled poly(Glu_4-Tyr_1) copolymers. (*A*, *C*, and *D*) The mean and SEM of two experiments are shown.

Two catalytically inert PTEN variants were created (PTEN;C124S and PTEN;D92A). Mutations of the corresponding residues in other phosphatases results in loss of enzymatic activity but allows for preservation of substrate binding and have been termed "substrate trapping" (34). As predicted, and as previously published (22, 23), the PTEN substrate-trapping mutants lack catalytic activity (Fig. 2 *C* and *D* and data not shown). Nonetheless, both PTEN;C124S and PTEN;D92A retained a partial ability to induce cells to accumulate in G_1 (Fig. 2*A* and data not shown). These data suggest that sequestration of phosphorylated substrates might be sufficient for cell-cycle inhibition by PTEN. PTEN;C124S can induce an accumulation of PtdIns-3,4,5-P_3 in cells, suggesting that there may be stable binding to this substrate (23). On the other hand the C124S mutant is defective in regulating cell motility (26). Thus, these data raised the possibility that the ability of PTEN to induce a G_1 block was distinct from cell motility control and suggested a relationship between the ability of PTEN to interact with a PtdIns substrate and the ability to arrest cells in G_1.

Protein Phosphatase Activity Is Not Sufficient for Induction of a G_1 Block by PTEN. We next compared the G129R mutant to a second mutant in which the same codon is affected (G129E). G129E is encoded by a *PTEN* allele found in the germ-line *PTEN* gene of two families afflicted with CD (35). Others have found that protein phosphatase activity of PTEN;G129E is equivalent to the wild-type protein (22) and that PTEN;G129E is comparable to wild-type PTEN in its ability to inhibit cell spreading (26). Indeed, in our assays we likewise see retention of protein phosphatase activity (Fig. 3*D*). In five preparations of GST-PTEN;G129E, activity varied from 30% to 70% of the wild-type activity (Fig. 3*D* and data not shown). However, when produced in 786-O cells, this PTEN mutant did not induce a G_1 block (Fig. 3*A*). Thus, both G129E and G129R failed to induce a G_1 block, even though the former retains protein phosphatase activity (Fig. 3*D*). We next asked whether the G129E mutant might be defective in dephosphorylating [^{3}H]IP_4, as a measure of lipid phosphatase activity. Indeed, although PTEN;WT catalyzed the dephosphorylation of [^{3}H]IP_4, neither G129R nor G129E had measurable activity in this assay (Fig. 3*C*). Thus, the ability of PTEN to induce a cell-cycle block correlated best with its ability to dephosphorylate a lipid substrate, and preservation

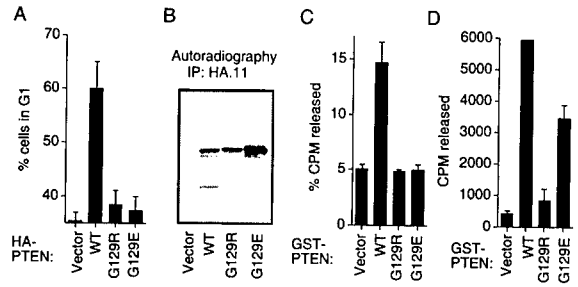

FIG. 3. CD mutant G129E preserves protein phosphatase activity but lacks inositol phosphatase activity and is incapable of inducing a G_1 block. (*A*) Comparison of wild-type PTEN and the mutants G129R and G129E in the cell-cycle assay. 786-O cells were cotransfected with pCD19 and plasmids encoding the indicated proteins and analyzed as in Fig. 1*A*. (*B*) Comparison of the expression of wild-type PTEN and the mutants G129R and G129E in 786-O cells. 786-O cells were transfected with plasmids encoding the indicated proteins. Anti-HA immunoprecipitates of protein extracts prepared from metabolically labeled cells were separated by gel electrophoresis and subject to fluorography. (*C*) Inositol phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were analyzed as in Fig. 2*C*. (*D*) Protein tyrosine phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were analyzed as in Fig. 2*D*. (*A*, *C*, and *D*) The mean and SEM of two experiments are shown.

of protein phosphatase activity, as measured *in vitro*, was not sufficient for the induction of a G_1 block.

Akt Is Downstream of PTEN. Thus, our data suggested that the ability of PTEN to regulate cell-cycle progression was dependent upon its ability to dephosphorylate PtdIns-3,4,5-P_3 and raised the possibility that the regulation of downstream targets of phosphatidylinositol 3-kinase might be critical for PTEN-mediated cell-cycle control. One such effector is the protein product of the protooncogene *AKT*. We next sought to determine whether PTEN might down-regulate Akt kinase activity. U2-OS cells were transiently transfected with an empty vector plasmid or a plasmid encoding T7-epitope tagged Akt along with the backbone vector plasmid or plasmids encoding HA-PTEN or PTEN;G129E. Akt was recovered by anti-T7 immunoprecipitation and used to phosphorylate a polypeptide substrate in the presence of [γ-^{32}P]ATP. PTEN efficiently down-regulated Akt kinase activity, whereas PTEN;G129E did not (Fig. 4 *A* and *B*). Identical results were obtained when this experiment was carried out in 786-O cells, which lack PTEN protein (data not shown). Thus, PTEN can negatively regulate Akt kinase activity.

We next asked whether Akt or a myristoylated form of Akt could override a PTEN-induced G_1 block. 786-O cells were transiently transfected with pCD19 and plasmids encoding either empty vector or wild-type PTEN and either empty pLNCX vector or pLNCX plasmids encoding Akt or the indicated derivatives. Although wild-type Akt had a minimal effect on the PTEN-induced G_1 block, a myristoylated form of Akt that is targeted to the membrane independently of PtdIns-3,4,5-P_3 overcame a PTEN block. In contrast, kinase-inactive versions of both Akt and Myr-Akt were unable to override PTEN (Fig. 4*C*). PTEN levels were unchanged by overproduction of Akt or the indicated derivatives (data not shown). These data suggest that PTEN-mediated cell-cycle inhibition depends on negative regulation of the phosphatidylinositol 3-kinase/Akt signaling pathway.

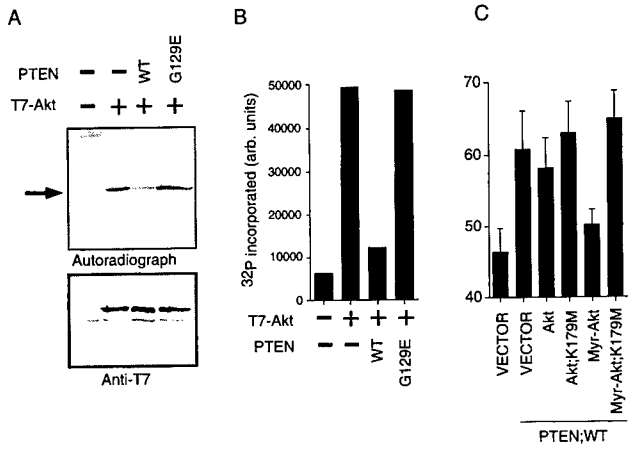

FIG. 4. PTEN inhibits Akt kinase activity and an activated form of Akt can override a PTEN induced G_1 block. (*A*) Inhibition of Akt kinase by wild-type PTEN. U2-OS cells were transfected with plasmids encoding the indicated proteins. After transfection, anti-T7 immunoprecipitates were prepared and used to phosphorylate a GST-peptide substrate *in vitro*. Autoradiography (*Upper*) and anti-T7 immunoblot (*Lower*) of the same membrane are shown. The black arrow indicates the position of the substrate. Results are representative of two experiments. (*B*) Quantitation of ^{32}P incorporation in *A* with a PhosphoImager. (*C*) Myr-Akt overrides a PTEN-induced G_1 block. 786-O cells were transiently cotransfected with pCD19 and plasmids encoding PTEN with empty vector (pLNCX) or with plasmids encoding the indicated Akt proteins. After transfection the cells were analyzed as in Fig. 1*A*. The mean and SEM of two experiments are shown.

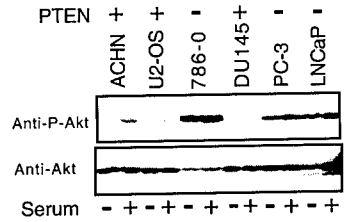

FIG. 5. Cells lacking PTEN protein contain high levels of Akt, phosphorylated on Ser-473. The indicated cells lines were serum-deprived for 24 h in duplicate. One p100 plate from each pair was refed with fresh medium containing 10% fetal calf serum for 90 min, after which protein extracts were prepared and subject to immunoblot analysis with an antibody directed at Akt phospho-Ser-473 (*Upper*) or an antibody that recognizes Akt independent of its phosphorylation status (*Lower*).

If Akt is a critical downstream target of PTEN tumor suppressor function, tumors or cell lines that lack PTEN protein might be predicted to harbor elevated levels of Akt kinase activity. As a measure of endogenous Akt activity, we subjected cell extracts to immunoblotting with an antibody specific to phospho-Ser-473 on Akt. Phosphorylation of this residue along with Thr-308 is required for Akt kinase activity (36). Protein extracts from duplicate plates of a panel of either PTEN+ or PTEN− cells were prepared after serum deprivation or after 90 min of serum stimulation and immunoblotted with the phospho-specific antibody. Protein extracts prepared from cells that contain PTEN protein by immunoblot, ACHN, DU145, and U2-OS (Fig. 1*D* and data not shown) were found to have little phosphorylated Akt (Fig. 5 *Upper*). In contrast, cells that lack PTEN protein, 786-O, LNCaP, and PC-3 (Fig. 1*D* and data not shown), had increased levels of phosphorylated Akt that was not down-regulated by serum withdrawal (Fig. 5 *Upper*). Levels of total Akt-1 protein in these cells were comparable (Fig. 5 *Lower*). Thus, loss of PTEN, in these tumor cell lines, was specifically correlated with a deregulation of the phosphorylated form of Akt.

DISCUSSION

Reintroduction of PTEN into 786-O cells led to an increase in the proportion of cells in G_1. Whether this represents a block in G_1 or a prolongation of G_1 awaits further study. Every human derived PTEN mutation we have tested to date has been defective in this cell-cycle assay. Thus, it is clear that the G_1 arrest is a reflection of a critical function of PTEN that is tightly linked to its function as a tumor suppressor protein.

What are the underlying mechanisms by which PTEN might exert control over progression through G_1? First, this function appears to be quite distinct from PTEN-mediated inhibition of cell spreading and cell motility. PTEN inhibition of cell motility and adhesion was observed in cells containing endogenous PTEN, was observed in cells only when grown on a fibronectin matrix, and was not observed with the PTEN;C124S mutant. In contrast, PTEN overexpression induced a G_1 block in cells lacking, but not in cells producing, PTEN protein, could be induced in cells plated on poly-(L-lysine)-coated plates, and was partially induced by the C124S mutant. Finally, PTEN;G129E clearly separates these two functions, because this mutant inhibits cell spreading comparably to the wild-type protein (26) but is incapable of arresting 786-O cells in G_1 (Fig. 3). Thus, these data suggest that the alterations in cell-cycle profile are not an indirect consequence of inhibition of cellular adhesion. Furthermore, that PTEN;G129E preserves protein phosphatase activity and can negatively regulate cell spreading yet is linked to the development of CD suggests that these functions are not sufficient for suppression of the CD phenotype. On the other hand, PTEN;G129E is defective in the cell-cycle assay. Thus, this

biological assay might be indicative of PTEN interactions with critical physiological substrates, regulation of which might be essential for preventing the onset of CD. Indeed, this mutant is defective in catalyzing the dephosphorylation of $[^3H]IP_4$, suggesting that *in vivo* dephosphorylation of PtdIns-3,4,5-P_3 is a critical requirement for PTEN-mediated suppression of CD. Similar results were recently reported by two groups (37, 38). Thus, we propose that the development of CD is linked to a loss of PTEN lipid phosphatase activity and to the subsequent deregulation of the cell cycle.

In support of this notion, mice that have only a single intact *PTEN* allele develop a syndrome not unlike CD and are phenotypically characterized by hyperplasia and dysplasia of the skin, gastrointestinal tract, and prostate (27). Notably, in the prostate of *PTEN*+/− mice both Ki-67 staining and the mitotic index indicated a significant increase in the proportion of cells that were proliferating when compared with wild-type mice. These data indicate that in murine prostate epithelial cells, the cell-cycle distribution has been altered by PTEN loss (27). In addition, wide-spread increased bromodeoxyuridine incorporation is found in PTEN mutant embryos at day 7.5 to 8.5 when compared with wild-type embryos (39). Finally, PTEN-mediated growth inhibition was recently linked to induction of a G_1 arrest rather than induction of apoptosis in glioblastoma cell lines (37).

Thus, our data and the data describing proliferative abnormalities in PTEN+/− mice suggest that PTEN plays a role in cell-cycle control. This function in turn appears to require lipid phosphatase activity. These data led us to ask whether Akt, a known downstream target of phosphatidylinositol 3-kinase that is activated by PtdIns-3,4,5-P_3, could act downstream of PTEN. First, we found that wild type but not the G129E mutant of PTEN could inhibit Akt kinase activity, suggesting again that PTEN protein phosphatase activity is not sufficient for inhibition of Akt. We next asked whether Akt could override a PTEN-mediated cell-cycle block. In keeping with the role of PTEN in limiting the availability of PtdIns-3,4,5-P_3, wild-type Akt was ineffective in this assay, whereas expression of a myristoylated form of Akt led to an override of the PTEN cell-cycle block. These data support the notion that Akt is an important downstream target of PTEN regulation. Similar conclusions have been reached by studying the effect of PTEN loss on murine fibroblasts (39).

Inducible expression of phosphatidylinositol 3-kinase can induce DNA synthesis in the absence of serum and is thus sufficient for initiation of this process (40). Akt and phosphatidylinositol 3-kinase can activate a number of downstream targets that may be involved in the regulation cell proliferation (41). The ribosomal protein $p70^{S6K}$ regulates the increased translation of a subset of mRNA species thought to be important for cell-cycle progression and, indeed, inactivation of $p70^{S6K}$ function leads to an arrest of cells in G_1 (42, 43). Likewise, Akt can induce phosphorylation of 4E-BP1, an event that leads to 4E-BP1 dissociation from the eukaryotic initiation factor eIF4E and a subsequent "disinhibition" of translation (44). eIF4E when overproduced transforms NIH 3T3 cells and, thus, like Akt, is an attractive tumor suppressor target (45). Which component(s) in the phosphatidylinositol 3-kinase/Akt pathway are rate-limiting for S phase entry is not known.

Akt is also an important component of a cell-survival signaling pathway and in this capacity can phosphorylate and inactivate the Bcl-2 family member BAD, rendering it incapable of blocking Bcl-2 or Bcl-X_L activity (41, 46, 47). Recent data has shown that PTEN−/− cells are resistant to apoptotic stimuli and that regulation of Akt is abnormal in PTEN-deficient fibroblasts (39). To date, we have not seen an increase in apoptosis upon reintroduction of PTEN into 786-O cells (Table 1). Among many possibilities, these data may indicate that additional inactivating mutations have been sustained in this cell line, rendering it incapable of responding to a variety of apoptotic signals. Finally, increases in phosphatidylinositol 3-kinase activity do not uniformly result in protection from apoptosis. In certain circumstances, phosphatidylinositol 3-kinase activity can induce cell-cycle progression and, in fact, promote apoptosis (40) (R. Narsimhan and T.M.R., unpublished data). The notion that PTEN may regulate both cell-cycle and cell-survival functions is strikingly similar to the tumor suppressor functions imputed to p53. Thus PTEN, like p53, may serve to coordinate these activities, although possibly in response to different sets of signals (48).

If Akt is a critical downstream target of PTEN, tumor cells that lack PTEN might be predicted to harbor excessive Akt activity. Immunoblots of protein extracts from cells that were characterized as PTEN− or PTEN+ (Fig. 1*D* and data not shown) were found to have a marked increase in the amount of Akt phosphorylated on Ser-473 (Fig. 5). This inverse correlation between PTEN and activated Akt again argues for a role for PTEN in Akt regulation in tumors. In this regard, inhibition of Akt activity by a dominant negative form of Akt blocks BCR-ABL transformation of murine myeloid cells and stable expression of antisense Akt2 reduces tumorigenicity of pancreatic cancer cell lines known to have amplified Akt (49, 50). Thus, inhibition of Akt family members in these settings does demonstrate antitumor activity. Whether activated Akt is necessary for the transformed phenotype of PTEN null tumors is a critical question. Nonetheless, our data raise the possibility that PTEN null tumors may be susceptible to Akt inhibition as a cancer treatment strategy.

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1. Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J. & Spurr, N. K. (1995) *Cancer Res.* **55,** 4800–4803.
2. Rasheed, B. K., McLendon, R. E., Friedman, H. S., Friedman, A. H., Fuchs, H. E., Bigner, D. D. & Bigner, S. H. (1995) *Oncogene* **10,** 2243–2246.
3. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., *et al.* (1997) *Nat. Genet.* **15,** 356–362.
4. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., *et al.* (1997) *Science* **275,** 1943–1947.
5. Li, D. M. & Sun, H. (1997) *Cancer Res.* **57,** 2124–2129.
6. Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., Bigner, D. D. & Bigner, S. H. (1997) *Cancer Res.* **57,** 4187–4190.
7. Liu, W., James, C. D., Frederick, L., Alderete, B. E. & Jenkins, R. B. (1997) *Cancer Res.* **57,** 5254–5257.
8. Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A. F. & Zeuthen, J. (1997) *Cancer Res.* **57,** 3660–3663.
9. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., Jen, J., Isaacs, W. B., Bova, G. S. & Sidransky, D. (1997) *Cancer Res.* **57,** 4997–5000.
10. Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K. L., Vinson, V. L., Gumpper, K. L., *et al.* (1997) *Cancer Res.* **57,** 5221–5225.
11. Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., Li, J., Parsons, R. & Ellenson, L. H. (1997) *Cancer Res.* **57,** 3935–3940.
12. Kohno, T., Takahashi, M., Manda, R. & Yokota, J. (1998) *Genes Chromosomes Cancer* **22,** 152–156.
13. Kong, D., Suzuki, A., Zou, T. T., Sakurada, A., Kemp, L. W., Wakatsuki, S., Yokoyama, T., Yamakawa, H., Furukawa, T., Sato, M., *et al.* (1997) *Nat. Genet.* **17,** 143–144.
14. Okami, K., Wu, L., Riggins, G., Cairns, P., Goggins, M., Evron, E., Halachmi, N., Ahrendt, S. A., Reed, A. L., Hilgers, W., *et al.* (1998) *Cancer Res.* **58,** 509–511.

15. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C. & Parsons, R. (1997) *Nat. Genet.* **16,** 64–67.
16. Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C. F., Fryns, J. P., Sijmons, R. H., Woods, D. G., *et al.* (1997) *Hum. Mol. Genet.* **6,** 1383–1387.
17. Tsou, H. C., Ping, X. L., Xie, X. X., Gruener, A. C., Zhang, H., Nini, R., Swisshelm, K., Sybert, V., Diamond, T. M., Sutphen, R. & Peacocke, M. (1998) *Hum. Genet.* **102,** 467–473.
18. Marsh, D. J., Dahia, P. L., Coulon, V., Zheng, Z., Dorion-Bonnet, F., Call, K. M., Little, R., Lin, A. Y., Eeles, R. A., Goldstein, A. M., *et al.* (1998) *Genes Chromosomes Cancer* **21,** 61–69.
19. Chuang, J. Z., Lin, D. C. & Lin, S. (1995) *J. Cell Biol.* **128,** 1095–1109.
20. Ungewickell, E., Ungewickell, H., Holstein, S. E., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L. E. & Eisenberg, E. (1995) *Nature (London)* **378,** 632–635.
21. Tonks, N. K. & Neel, B. G. (1996) *Cell* **87,** 365–368.
22. Myers, M. P., Stolarov, J., End, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. & Tonks, N. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9052–9057.
23. Maehama, T. & Dixon, J. E. (1998) *J. Biol. Chem.* **273,** 13375–13378.
24. Furnari, F. B., Lin, H., Huang, H. S. & Cavenee, W. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12479–12484.
25. Cheney, I. W., Johnson, D. E., Vaillancourt, M. T., Avanzini, J., Morimoto, A., Demers, G. W., Wills, K. N., Shabram, P. W., Bolen, J. B., Tavtigian, S. V. & Bookstein, R. (1998) *Cancer Res.* **58,** 2331–2334.
26. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. & Yamada, K. M. (1998) *Science* **280,** 1614–1617.
27. Di Cristafano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P. P. (1998) *Nat. Genet.* **19,** 348–355.
28. Sellers, W. R., Novitch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., Lassar, A. B. & Kaelin, W. G., Jr. (1998) *Genes Dev.* **12,** 95–106.
29. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
30. Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W. G., Jr. (1995) *Nat. Med.* **1,** 822–826.
31. Tedder, T. F. & Isaacs, C. M. (1989) *J. Immunol.* **143,** 712–717.
32. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning:A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
33. Myers, M. P. & Tonks, N. K. (1997) *Am. J. Hum. Genet.* **61,** 1234–1238.
34. Flint, A. J., Tiganis, T., Barford, D. & Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1680–1685.
35. Marsh, D. J., Coulon, V., Lunetta, K. L., Rocca-Serra, P., Dahia, P. L., Zheng, Z., Liaw, D., Caron, S., Duboue, B., Lin, A. Y., *et al.* (1998) *Hum. Mol. Genet.* **7,** 507–515.
36. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. & Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551.
37. Furnari, F. B., Huang, H. J. & Cavenee, W. K. (1998) *Cancer Res.* **58,** 5002–5008.
38. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P. & Tonks, N. K. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 13513–13518.
39. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P. & Mak, T. W. (1998) *Cell* **95,** 29–39.
40. Klippel, A., Escobedo, M. A., Wachowicz, M. S., Apell, G., Brown, T. W., Giedlin, M. A., Kavanaugh, W. M. & Williams, L. T. (1998) *Mol. Cell. Biol.* **18,** 5699–5711.
41. Downward, J. (1998) *Curr. Opin. Cell Biol.* **10,** 262–267.
42. Peterson, R. T. & Schreiber, S. L. (1998) *Curr. Biol.* **8,** R248–R250.
43. Lane, H. A., Fernandez, A., Lamb, N. J. & Thomas, G. (1993) *Nature (London)* **363,** 170–172.
44. Gingras, A. C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N. & Hay, N. (1998) *Genes Dev.* **12,** 502–513.
45. Lazaris-Karatzas, A., Montine, K. S. & Sonenberg, N. (1990) *Nature (London)* **345,** 544–547.
46. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. & Nunez, G. (1997) *Science* **278,** 687–689.
47. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M. E. (1997) *Cell* **91,** 231–241.
48. Evan, G. & Littlewood, T. (1998) *Science* **281,** 1317–1321.
49. Skorski, T., Bellacosa, A., Nieborowska-Skorska, M., Majewski, M., Martinez, R., Choi, J. K., Trotta, R., Wlodarski, P., Perrotti, D., Chan, T. O., *et al.* (1997) *EMBO J.* **16,** 6151–6161.
50. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K. & Testa, J. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3636–3641.

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Loss of PTEN Expression in Paraffin-embedded Primary Prostate Cancer Correlates with High Gleason Score and Advanced Stage[1]

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ABSTRACT

The tumor suppressor gene PTEN/MMAC-1/TEP-1 (referred to hereafter as PTEN) maps to chromosome 10q23 and encodes a dual specificity phosphatase. The PTEN protein negatively regulates cell migration and cell survival and induces a G_1 cell cycle block via negative regulation of the phosphatidylinositol 3′-kinase/protein kinase B/Akt signaling pathway. *PTEN* is frequently mutated or deleted in both prostate cancer cell lines and primary prostate cancers. A murine polyclonal antiserum was raised against a glutathione *S*-transferase fusion polypeptide of the COOH terminus of PTEN. Archival paraffin tissue sections from 109 cases of resected prostate cancer were immunostained with the antiserum, using DU145 and PC-3 cells as positive and negative controls, respectively. PTEN expression was seen in the secretory cells. Cases were considered positive when granular cytoplasmic staining was seen in all tumor cells, mixed when areas of both positive and negative tumor cell clones were seen, and negative when adjacent benign prostate tissue but not tumor tissue showed positive staining. Seventeen cases (15.6%) of prostate cancer were positive, 70 cases (64.2%) were mixed, and 22 cases (20.2%) were negative. Total absence of PTEN expression correlated with the Gleason score ($P = 0.0081$) and correlated more significantly with a Gleason score of 7 or higher ($P = 0.0004$) and with advanced pathological stage (American Joint Committee on Cancer stages T3b and T4; $P = 0.0078$). Thus, loss of PTEN protein is correlated with pathological markers of poor prognosis in prostate cancer.

INTRODUCTION

PTEN is a tumor suppressor gene that maps to the 10q23.3 interval (1–3). The protein product, PTEN, shares homology with the cytoskeletal protein tensin and the secretory vesicle protein auxilin and also with dual specificity phosphatases. Indeed, recombinant PTEN exhibits activity against both phosphotyrosine- and phosphothreonine-containing protein substrates (4). Overexpression of PTEN suppresses tumor colony formation in certain cell lines and can suppress tumor formation in nude mice (5–7). PTEN overexpression can also negatively regulate cellular adhesion and cell mobility on fibronectin-coated plates (8). This activity may result from PTEN-mediated dephosphorylation of focal adhesion kinase. PTEN may also alter mitogen-activated protein kinase signaling (9).

PTEN can also act as a lipid phosphatase. Specifically, PTEN can dephosphorylate phosphotidylinositol 3,4,5-trisphosphate and phosphotidylinositol 3,4-trisphosphate, which are both direct products of PI3K[3] activity (10). We and others have shown that PTEN can inhibit cell cycle progression and induce a G_1 arrest. This function appears to require the lipid phosphatase activity of PTEN, resulting in the negative regulation of the PI3K/Akt signaling pathway (11–13). A significant increase in the level of the cell cycle kinase inhibitor $p27^{KIP1}$ occurs with concomitant decreases in G_1 cyclin-dependent kinase activity upon the introduction of PTEN into human glioblastoma U87MG cells, suggesting that p27 may be a target of the PTEN cell cycle arrest pathway (13). In keeping with these data, heterozygous loss of the murine *PTEN* gene (*mPTEN*) leads to an increase in the mitotic index and the Ki-67 staining index in the murine prostate (7). In addition, PTEN negatively regulates Akt-dependent cell survival (14–17). Akt is one of the key regulatory molecules involved in the protection of cells against apoptosis. These data support the idea that PTEN negatively regulates cell growth and/or proliferation through its ability to act as an *in vivo* phosphoinositide 3-phosphatase, thus negatively regulating the PI3K/Akt signaling pathway.

Germ-line mutations of *PTEN* have been detected in cases of Cowden disease and Bannayan-Zonana syndrome, two related hamartoma syndromes (18–20). Patients with Cowden disease have an elevated risk of various cancers, including breast and thyroid cancer. Alterations of the second *PTEN* allele have been demonstrated in gastrointestinal polyps in patients with Cowden disease (21).

Somatic alterations of *PTEN* are common in certain cell lines and in primary tumors including gliomas (22–25), melanoma (26, 27), and thyroid (28) and endometrial cancers (29, 30). On the other hand, somatic alterations are rare in breast (31) and renal cancer (32) and were not detected in a series of squamous carcinomas from the head and neck (33). *PTEN* mutations and allele loss at 10q23 appear to be a late event in glioblastoma, melanoma, and prostate cancer (22–26, 34). In contrast, *PTEN* alterations are more common in benign tumors than in malignant thyroid tumors (28) and also occur in a proportion of cases of endometrial hyperplasia, a precursor of endometrial carcinoma (35), suggesting that the genetic alteration may occur at an early stage in these tumors.

Prostate cancer is the most prevalent form of cancer in men in the Western world and is the second most common cause of male cancer deaths in the United States (36). Pathological stage and Gleason grade are important predictors of prognosis in patients with primary prostate cancer who undergo radical prostatectomy. Prostate cancer, however, is a remarkably heterogeneous disease. Distinguishing tumors associated with a poor outcome at the time of radical prostatectomy is problematic. The molecular mechanisms of prostate carcinogenesis remain poorly understood. LOH of 10q has been reported to occur in prostate cancer with a high frequency (30–60%; Refs. 37 and 38), and two distinct, commonly deleted regions have been identified at 10q22–q24 and 10q25, respectively, implying the presence of putative tumor suppressor genes at these loci (38). Homozygous deletions and somatic mutations of *PTEN* have been identified in prostate cell lines and tumor specimens (1, 2, 34, 39–45). Marked heterogeneity of *PTEN* alterations has been observed in metastatic prostate cancer tissues (43). Loss of PTEN expression is more frequently detected in xenografts of cell lines (34). *PTEN* may be inactivated by mechanisms other than gene deletion and mutations, including promoter methylation or translational modification (34). However, other groups failed

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[3] The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; LOH, loss of heterozygosity.; GST, glutathione *S*-transferase; HA, hemagglutinin; PIN, prostatic intraepithelial neoplasia.

to detect *PTEN* methylation in prostate, bladder, and renal cell cancer with LOH of 10q when a PCR-based assay was used (32, 40).

In this study, we wanted to assess the extent of loss of the PTEN protein in prostate cancer using immunohistochemistry. We analyzed the pattern of immunohistochemical staining in 109 cases of paraffin-embedded resected prostate cancer using a murine polyclonal antibody to PTEN. Detection of PTEN protein was correlated with the Gleason score and the pathological stage of the tumor, known prognosticators in prostate cancer.

MATERIALS AND METHODS

Tissue Specimens. We used cases from a prostate database consisting of 128 paraffin-embedded prostate cancers that had been collected in the Department of Pathology, Beth Israel Deaconess Medical Center, West Campus, dating from 1990–1997. This database has been described previously (46–49). Nineteen cases were transurethral resection specimens. We used the remaining 109 radical prostatectomy specimens for this study. Follow-up data were available on 69 cases in the database, with a mean patient follow-up of 19.84 months. The pathological tumor (T) stage (American Joint Committee on Cancer; Ref. 50) and Gleason score were available in each case. Five of the 109 cases had been treated with preoperative total androgen ablation. For each case, a representative paraffin block was selected that contained both tumor and benign prostate tissue.

Processing of Cell Lines and Cell Blocks. DU145, PC-3, and LNCaP prostate cancer cell lines were obtained from the American Type Culture Collection. The U2-OS cell line was a generous gift of W. G. Kaelin (Dana-Farber Cancer Institute). DU145 cells contain one wild-type *PTEN* allele and a second variant allele (*M134L*). PC-3 cells have sustained a homozygous deletion of *PTEN*. LNCaP cells have a deletion of one allele and a mutation of the other *PTEN* allele, and the genetic state of *PTEN* has not been characterized in U2-OS cells. LNCaP and PC-3 cells were grown in RPMI 1640 supplemented with D-glucose, HEPES buffer, L-glutamine, PP_i, penicillin, streptomycin, and 10% fetal bovine serum. DU145 cells were maintained in DMEM supplemented with penicillin, streptomycin, and 10% fetal bovine serum, and U2-OS cells were maintained in DMEM supplemented with penicillin, streptomycin, and 10% fetal clone (HyClone). All cells were grown on P100 tissue culture dishes at 37°C. LNCaP and PC-3 cells were grown in a 5% CO_2 atmosphere, and DU145 and U2-OS cells were grown in a 10% CO_2 atmosphere. Cell pellets were created from DU145 and PC-3 cells, fixed in 10% formalin overnight, and then processed in the regular manner for pathology specimens to produce paraffin cell blocks.

Plasmids. A cDNA fragment of the *PTEN* gene encoding amino residues 239–403 was amplified by PCR using primers WRSO-56 (5′-GACTGGATCCATGTACTTTGAGTTCCCTCAGCC-3′) and WRSO-57 (5′-CGCGGAATTCTCAGACTTTTGTAATTTGTGTATGC-3′) from a cDNA library derived from human embryonic kidney 293 cells (51). The resulting PCR fragment was isolated, restricted with *Bam*HI and *Eco*RI, and ligated to similarly restricted pSG5L to produce pSG5L-PTEN (239–403). This cDNA was confirmed by sequencing. The insert from this plasmid was excised and ligated to *Bam*HI/*Eco*RI-restricted pGEX2T vector to produce pGEX2T-PTEN (239–403) plasmid.

Antibodies. Recombinant GST-PTEN (239–403) was produced in *Escherichia coli* and affinity-purified on glutathione-Sepharose beads by conventional methods (52). Mice were inoculated with 100 μg of GST-PTEN (239–403) mixed with Freund's complete adjuvant. Two weeks later, the mice received a subsequent boost of 100 μg of the purified protein in Freund's incomplete adjuvant. Immune sera (M1) was obtained by orbital sinus puncture.

***In Vitro* Translation, Immunoprecipitation, and Immunoblotting.** Full-length HA-tagged PTEN protein (HA-PTEN) was produced *in vitro* by coupled transcription and translation of the pSG5L-PTEN plasmid using the TnT kit (Promega, Madison, WI). Cell extracts were prepared in the following manner. Cells grown on P100 plates were washed twice with PBS and then lysed on the plate in 500 μl of TNN buffer [150 mM NaCl, 50 mM Tris (pH 7.4), and 0.5% NP40] at 4°C for 20 min. Collected extracts were then cleared by centrifugation at 14,000 rpm for 15 min. Immunoprecipitations of *in vitro* translated products were carried out at 4°C in NET-N buffer [120 mM NaCl, 10 mM EDTA (pH 8.0), 100 M Tris (pH 7.4), and 0.5% NP40] along with 5 μl of *in vitro* translated PTEN in 250 μl of NET-N. One μl of antiserum was used per immunoprecipitation experiment. Immune complexes were captured on protein A-Sepharose beads (30 μl of 1:1 beads), washed five times with NET-N, and boiled in 1× Laemmli sample buffer. Whole cell extracts or immunoprecipitates were separated by vertical gel electrophoresis on 7.5% gels. Proteins were transferred to Sequi-blot polyvinylidene difluoride membrane (Bio-Rad) by wet transfer in Towbin's buffer for 6–16 h. Immunoblots were blocked in TBS + 4% milk. M1 was used at a concentration of 1:10,000 in TBS + 4% milk. Alkaline phosphatase-conjugated goat antimouse antibody was the secondary antibody.

Immunohistochemistry. Five-μm sections were cut from the selected paraffin blocks of prostate tumor and the DU145 and PC-3 cell blocks, mounted on charged glass slides, baked at 60°C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses. Slides were immersed in 10 mM/liter citrate buffer (pH 6.0; Biogenex, San Ramon, CA) and microwaved in a 750 W oven inside a pressure cooker for 30 min. The slides were cooled at room temperature for 15 min and rinsed in tap water. A 1:2000 dilution of M1, the PTEN murine polyclonal antiserum, was applied for 32 min at 37°C. An automated processor (Ventana ES; Ventana Medical Systems, Tuscon, AZ) was used to incubate the slides in blocker (10% normal goat serum and 10% normal horse serum in Ventana diluent) for 8 min, followed by an incubation in secondary antibody conjugated to an avidin-biotin peroxidase complex (antirabbit and antimouse). Finally, 3,3′-diaminobenzidine was used as a substrate to detect bound antibody complex. The slides were counterstained with hematoxylin. Standardization of the incubation and development times allowed an accurate comparison of expression levels in all cases.

Analysis of Immunohistochemical Staining. Positive cases were defined by the presence of granular, crisp cytoplasmic staining, as seen in the DU145 positive control samples. The cases were initially divided into three groups: (*a*) positive (the entire tumor showed staining); (*b*) mixed (both positive and negative cells/glands were present); and (*c*) negative (no staining was seen in the represented tumor). The grading of PTEN expression was performed without knowledge of the Gleason score or pathological stage. The presence of positive staining in PIN was noted. The cases were then divided into those that showed positive staining (positive and mixed groups) and those with a total absence of staining (negative group).

Statistical Analysis. We tested for associations between PTEN expression and Gleason score or pathological stage of disease using the Mann-Whitney nonparametric *U* test, the c^2 test, or Fisher's exact test, as appropriate. All calculations were performed using StatView 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Characterization of the Antibody. A murine polyclonal antiserum (M1) was raised against a protein chimera encoding GST and PTEN amino acid residues 237–403. HA-PTEN was produced by *in vitro* translation and subjected to immunoprecipitation with M1. Purified anti-HA antibody and the M1 preimmune sera served as positive and negative controls, respectively. Both the M1 antiserum and the anti-HA antibody immunoprecipitated HA-PTEN, whereas the nonimmune serum did not (Fig. 1, *left panel*). To determine whether M1 might specifically recognize the endogenous PTEN protein, whole cell protein extracts were prepared from U2-OS osteosarcoma cells and DU145 and PC-3 prostate carcinoma cells, separated by electrophoresis, and subjected to immunoblotting with M1 antiserum (Fig. 1, *right panel*). M1 recognized a protein species of approximately M_r 58,000 that is present in DU145 and U2-OS cells but is absent in PC-3 cells. PC-3 cells have sustained a biallelic deletion of the *PTEN* gene, whereas DU145 contains a wild-type *PTEN* allele and an allele harboring a missense change at codon 134 (M134L). This protein species migrates slightly faster than the *in vitro* translated HA-PTEN. Taken together, these data indicate that the recognized protein is endogenous PTEN.

PTEN Expression in Human Prostate Cancer Cell Lines. Next we asked whether M1 was capable of recognizing PTEN by immu-

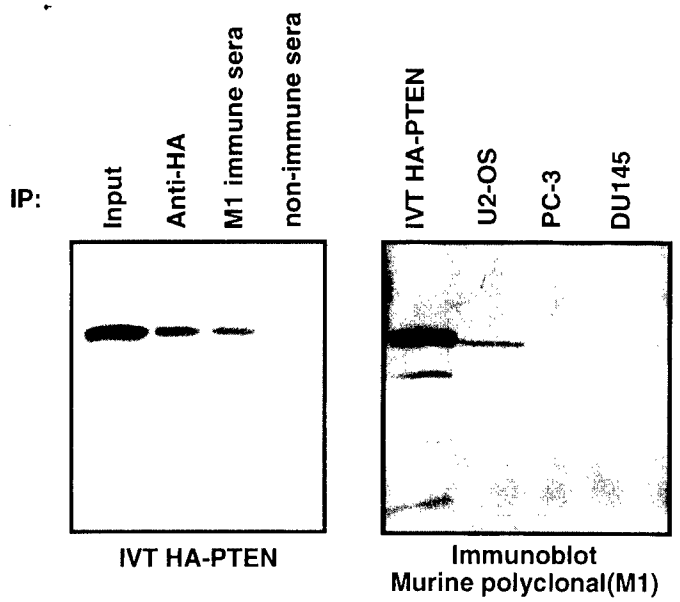

Fig. 1. Immunoprecipitation of *in vitro* translated HA-PTEN with the murine polyclonal antisera M1. HA-PTEN was produced by *in vitro* translation and immunoprecipitated with either anti-HA antibody, murine polyclonal antisera M1, or nonimmune murine antisera, as indicated (*left panel*). The input is 5 μl of *in vitro* translated HA-PTEN loaded in sample buffer. Immunoblot detection of endogenous PTEN was performed using murine polyclonal antisera M1.Whole cell extracts (150 μg) prepared from U2-OS cells, PC-3 cells, and DU145 cells (as indicated) and HA-PTEN produced by *in vitro* translation were separated by gel electrophoresis, transferred to polyvinylidene difluoride membrane, and immunoblotted with murine polyclonal antisera M1 (*right panel*).

nohistochemistry. Cell blocks were prepared from DU145 and PC-3 cells that were grown in culture. M1 was then used to detect PTEN in 5-μm sections from these paraffin blocks by immunohistochemical means. Strong positive granular cytoplasmic staining was detected in DU145 cells, but PC-3 cells were negative (Fig. 2, *A* and *B*). Thus, sections of cell blocks of DU145 and PC-3 cell lines served as positive and negative controls, respectively. Preimmune serum also served as a negative control.

Patient Databases and Tumor Characteristics. The mean patient age at the time of surgery was 65.2 ± 8.4 years, with an age range of 40–86 years. The Gleason score of the tumors ranged from 4–9 with the following frequency: (*a*) Gleason score = 4, 4 tumors; (*b*) Gleason score = 5, 9 tumors; (*c*) Gleason score = 6, 17 tumors; (*d*) Gleason score = 7, 58 tumors; (*e*) Gleason score = 8, 12 tumors; and (*f*) Gleason score = 9, 9 tumors. The median Gleason score was 7. The cases were then subdivided into two groups: (*a*) those with a Gleason score < 7 (30 cases); and (*b*) those with a Gleason score ≥ 7 (79 cases; Table 1). The cases were divided into two groups: (*a*) those with either organ-confined disease or disease extends through into the prostate capsule (T1–T3a; 83 cases); and (*b*) those with seminal vesicle involvement or metastases to the lymph nodes (T3b and T4; 26 cases; Table 2).

PTEN Expression in Human Prostate Tissue. Benign prostate epithelium showed positive staining for PTEN with granular cytoplasmic staining observed in the prostatic secretory cells. PIN was present in the selected slides in 58 cases, and all cases showed positive staining (Fig. 3, *A1* and *A2*). The cases were initially divided into three groups: (*a*) positive (the entire tumor showed staining); (*b*) mixed (both positive and negative cells/glands were present); and (*c*) negative (no staining was seen in the represented tumor). Heterogeneous staining of the tumors was present. Seventeen cases (15.6%) were positive (Fig. 3, *B1* and *B2*). Seventy cases (64.2%) showed a mixed staining pattern. Specifically, there were areas of tumor that stained positively, whereas other areas of tumor showed negative staining (Fig. 3, *C1* and *C2*). The remaining 22 tumors (20.2%) were negative (Fig. 3, *D1* and *D2*). The cases were subsequently divided into those that showed positive staining (positive and mixed groups) and those with a total absence of staining (negative group). The results of PTEN expression in each group were compared.

Correlation of PTEN Expression with Gleason Score and Pathological Stage. Loss of PTEN expression correlated significantly with increasing Gleason score ($P = 0.0081$), and when cases were divided into those with a Gleason score < 7 and those with a Gleason score ≥ 7, the correlation with a Gleason score ≥ 7 was highly significant ($P = 0.0004$). Loss of PTEN expression also correlated with advanced disease (pathological tumor stage T3c and T4; $P = 0.0078$). PTEN expression was seen in two of the five tumors where patients had undergone preoperative total androgen ablation.

Follow-up for the cohort was too short to give meaningful survival figures because only four deaths had occurred in the study group.

DISCUSSION

In this study, a murine polyclonal antiserum (M1) was raised against a protein chimera encoding GST and PTEN amino acid residues 237–403. Using this antiserum, the expression of the PTEN protein was determined by immunohistochemistry in 109 prostate cancers of varying grade and pathological stage. Whereas PTEN was expressed in all cases of PIN, the presumed precursor lesion of

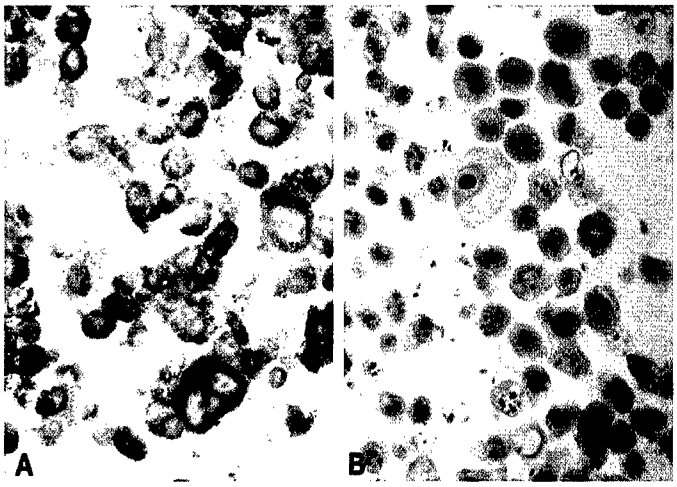

Fig. 2. PTEN expression in prostate cancer cell lines. Sections of cell blocks prepared from (*A*) DU145 cells (which contain one wild-type *PTEN* allele) or (*B*) PC-3 cells (which show a homozygous deletion of *PTEN*) were stained with M1 antiserum as described in "Materials and Methods." ×400.

Table 1 *Observed frequencies for PTEN expression and Gleason score*

	PTEN expression[a]		
Gleason score	Positive (%)	Mixed (%)	Negative (%)
<7	10 (9.2)	20 (18.3)	0 (0)
≥7	7 (6.4)	50 (45.9)	22 (20.2)

[a] Negative group *versus* positive plus mixed groups, $P = 0.0004$.

Table 2 *Observed frequencies for PTEN expression and pathological stage*

	PTEN expression[a]		
Pathological stage	Positive (%)	Mixed (%)	Negative (%)
T1–T3a	14 (12.8)	57 (52.3)	12 (11.0)
T3b and T4	3 (2.8)	13 (11.9)	10 (9.2)

[a] Negative group *versus* positive plus mixed groups, $P = 0.0078$.

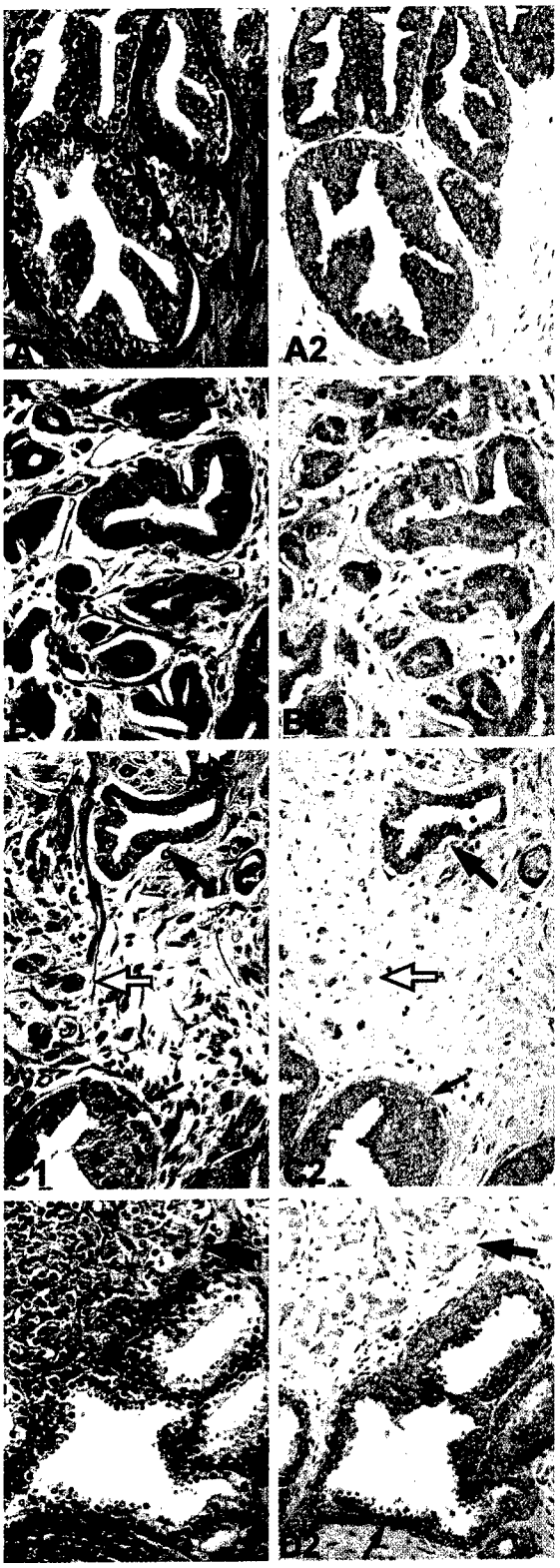

Fig. 3. PTEN expression in prostate tissue. *A*, PIN. *A1*, H&E-stained section of PIN. *A2*, positive staining for PTEN in PIN. *B*, prostate cancer (Gleason score, 3 + 3 = 6). *B1*, H&E-stained section. *B2*, positive staining for PTEN in prostate cancer. *C*, an example of heterogeneous expression of PTEN in a case of prostate cancer (Gleason score, 3 + 5 = 8). *C1*, prostate cancer. Gleason grade 3 cancer (*large black arrow*), Gleason grade 5 cancer (*open arrow*), and PIN (*short black arrow*). H&E-stained section. *C2*, Gleason grade 3 prostate cancer stains positively for PTEN (see *large black arrow*). In contrast, Gleason grade 5 prostatic carcinoma is negative for PTEN expression (*open arrow*). PIN shows positive staining for PTEN (*short black arrow*). *D*, benign prostate acinus with surrounding prostate cancer (Gleason score, 4 + 3 = 7). *D1*, H&E-stained section. *Small arrow*, benign prostatic glandular epithelium; *large arrow*, prostatic adenocarcinoma). *D2*, positive staining for PTEN in benign prostate tissue and absence of staining for PTEN in prostate cancer. ×200. All tissue sections were processed as described in "Materials and Methods."

prostate cancer (53), total loss of expression of PTEN was found in 20.2% of the prostate cancers. In an additional 70 of 109 cases (64.2%), there was a mixed pattern of staining, with areas of tumor with positive staining and other areas that were negative for PTEN. A mixed pattern of staining for PTEN was also recently found in glioma (54). Finally, 15.6% of tumors appeared to have homogenous positive staining for PTEN.

In this data set, complete PTEN loss was found to correlate significantly with the presence of high-stage disease (pathological stage T3b and T4; $P = 0.0078$). Indeed, *PTEN* mutations and allele loss at 10q23 have been reported to occur as a late event in most, albeit not all, tumors, including prostate cancers (22–26, 28, 34, 35). We also found that loss of PTEN expression correlated significantly with increasing grade of prostate cancer, *i.e.,* Gleason score ($P = 0.0081$). When cases were divided into those with a Gleason score < 7 or ≥ 7, loss of PTEN expression correlated significantly with a Gleason score ≥ 7 ($P = 0.0004$). A cut point between Gleason score 6 and 7 has previously been recommended when compression of the Gleason score is required (55). Similarly, *PTEN* is altered in high-grade gliomas, but not in low-grade gliomas (22, 23). On the other hand, *PTEN* alteration occurs in all three grades of endometrial cancer (29, 30), and mutation of a germ-line *PTEN* allele predisposes carriers to breast and thyroid cancer in humans and to a number of malignancies including prostate cancer in mice (7, 18, 20, 56). Thus, PTEN appears to play a role in the initiation of certain tumors, including a murine form of prostate cancer, and may play a role in the progression of other tumors such as gliomas and prostate cancer. Although seemingly paradoxical, the role of PTEN loss as an initiating event *versus* its role as an agent of progression might arise from fundamental differences between tissues with respect to the order of addition of various oncogenic events. For example, the human adult male prostate epithelial cell might not tolerate loss of PTEN unless the loss was first preceded by a permissive mutational event. On the other hand, this paradox, at least with respect to the prostate, might simply reflect upon our current ability, or lack thereof, to detect certain PTEN mutational events. Indeed, in our data set, the vast majority (85%) of tumors had a portion of the tumor in which PTEN staining was absent, in keeping with the marked heterogeneity of *PTEN* alterations that has been reported previously in metastatic prostate cancer samples (43). If it is the PTEN-null portion of the tumor that is destined to become the predominant metastatic clone, then the apparent lack of PTEN mutations in such organ-confined tumors might simply result from a lack of detection by conventional methodologies.

PTEN alterations have also been described in prostate cancer cell lines, xenografts, and tumors (1, 2, 34, 39–44). The true number of inactivating events is likely to be greater because the presence of sequence mutations in promoter/regulatory regions was not excluded by these studies. Of interest, there has been no evidence of *PTEN* promoter methylation in prostate cancers or bladder and renal cancers with 10q LOH using a DNA-based assay (32, 40). However, in certain prostate cancer cells, *PTEN* mRNA was restored after treatment with the demethylating agent 5-azadeoxycytidine (34). It is possible that methylation of a transcription factor for *PTEN* may play a role in the regulation of the gene.

Although we did not assess the genetic status of *PTEN* in our cases, loss of expression as assessed by immunohistochemistry might reflect a majority of the possible mechanisms resulting in PTEN inactivation. These would include direct inactivation by homozygous deletion, nonsense mutation, certain internal deletions, and promoter methylation or indirect inactivation such as loss of a PTEN-directed transcription factor or posttranscriptional modification, such as that which occurs with cdc25, another dual specificity phosphatase (57). Mis-

sense mutations, which do not grossly destabilize the protein product, would not be accounted for by immunohistochemistry.

PTEN appears to function, at least in part, by acting to brake cell cycle progression (11–13). We and others (11, 12) have previously demonstrated that this function appears to require PTEN lipid phosphatase activity, suggesting that cell cycle regulation may result from inhibition of the PI3K pathway. We further demonstrated that activated forms but not wild-type forms of the proto-oncogene Akt were capable of overriding a PTEN-mediated cell cycle block, raising the possibility that Akt might be an important downstream target of PTEN with respect to cell cycle progression (12). Similar conclusions have been reached with respect to the function of PTEN as a regulator of apoptosis or cell survival (14–17, 58). These data, taken together, suggest the possibility that targeted inhibition of the PI3K/Akt pathway might be of therapeutic value in patients with prostate cancer. We and others (12, 59) have also shown that cell lines and tumors in which PTEN is lost have elevated levels of activated Akt. Thus, loss of immunohistochemical detection of PTEN might predict for the presence of activated Akt and, in turn, might become useful as a factor predictive of success for therapies directed against this pathway. In general, this type of predictive factor, such as the estrogen receptor, which can predict for the efficacy of a given therapy, such as tamoxifen, has great clinical utility because it directly impacts treatment decisions.

Our results support the candidacy of PTEN as a tumor suppressor gene in prostate cancer progression. Indeed, loss of PTEN expression may be an important negative prognostic indicator. We are currently working on the development of well-characterized rabbit polyclonal or murine monoclonal antibodies that would provide unlimited amounts of antibody capable of reacting with formalin-fixed tissue. It is possible that immunohistochemistry may be the optimal method for evaluating the functional status of *PTEN* because it would detect a loss of PTEN induced by a majority of the mechanisms through which gene products are inactivated.

REFERENCES

1. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., *et al.* Identification of a candidate tumour suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., *15:* 356–362, 1997.
2. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., *et al. PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science (Washington DC), *275:* 1943–1947, 1997.
3. Li, D. M., and Sun, H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. Cancer Res., *57:* 2124–2129, 1997.
4. Myers, M. P., Stolarov, J., End, C., Li, J., Wang, S. I., Wigler, M. H., *et al.* PTEN, the tumor supressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl. Acad. Sci. USA, *94:* 9052–9057, 1997.
5. Furnari, F. B., Lin, H., Huang, H. S., and Cavenee, W. K. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc. Natl. Acad. Sci. USA, *94:* 12479–12484, 1997.
6. Cheney, I. W., Johnson, D. E., Vaillancourt, M. T., Avanzini, J., Morimoto, A., Demers, G. W., *et al.* Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated *MMAC1/PTEN* gene transfer. Cancer Res., *58:* 2331–2334, 1998.
7. Di Cristafano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. PTEN is essential for embryonic development and tumor suppression. Nat. Genet., *19:* 348–355, 1998.
8. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. Inhibition of cell migration, spreading, and focal adhesion by tumor suppressor PTEN. Science (Washington DC), *280:* 1614–1617, 1998.
9. Gu, J., Tamura, M., and Yamada, K. M. Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. J. Cell Biol., *143:* 1375–1383, 1998.
10. Machama, T., and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-triphosphate. J. Biol. Chem., *273:* 13375–13378, 1998.
11. Furnari, F. B., Huang, H. J., and Cavenee, W. K. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G_1 growth arrest in glioma cells. Cancer Res., *58:* 5002–5008, 1998.
12. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., *et al.* Regulation of G_1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA, *96:* 2110–2115, 1999.
13. Li, D., and Sun, H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G_1 cell cycle arrest in human glioblastoma cells. Proc. Natl. Acad. Sci. USA, *95:* 15406–15411, 1998.
14. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell, *95:* 29–39, 1998.
15. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA, *95:* 15587–15591, 1998.
16. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., *et al.* The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc. Natl. Acad. Sci. USA, *95:* 13513–13518, 1998.
17. Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N., *et al.* The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res., *58:* 5667–5672, 1998.
18. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., *et al.* Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet., *16:* 64–67, 1997.
19. Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., *et al.* Germline mutations in the *PTEN/MMAC1* gene in patients with Cowden disease. Hum. Mol. Genet., *6:* 1383–1387, 1997.
20. Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J., *et al.* Germline mutations in *PTEN* are present in Bannayan-Zonana syndrome. Nat. Genet., *16:* 333–334, 1997.
21. Chi, S. G., Kim, H. J., Park, B. J., Min, H. J., Park, J. H., Kim, Y. W., *et al.* Mutational abrogation of the *PTEN/MMAC1* gene in gastrointestinal polyps in patients with Cowden disease. Gastroenterology, *115:* 1084–1089, 1998.
22. Wang, S. I., Puc, J., Li, J., Bruce, J. N., Cairns, P., Sidransky, D., *et al.* Somatic mutations of PTEN in glioblastoma multiforme, Cancer Res., *57:* 4183–4186, 1997.
23. Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., *et al. PTEN* gene mutations are seen in high-grade but not in low-grade gliomas. Cancer Res., *57:* 4187–4190, 1997.
24. Liu, W., James, C. D., Frederick, L., Alderete, B. E., and Jenkins, R. B. PTEN/MMAC1 mutations and EGFR amplification in glioblastomas. Cancer Res., *57:* 5254–5257, 1997.
25. Duerr, E. M., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer-Puttlitz, B., Louis, D. N., *et al.* PTEN mutations in gliomas and glioneuronal tumors. Oncogene, *16:* 2259–2264, 1998.
26. Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A. F., and Zeuthen, J. Disruption of the *MMAC1/PTEN* gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res., *57:* 3660–3663, 1997.
27. Tsao, H., Zhang, X., Benoit, E., and Haluska, F. G. Identification of *PTEN/MMAC1* alterations in uncultured melanomas and melanoma cell lines. Oncogene, *16:* 3397–3402, 1998.
28. Dahia, P. L., Marsh, D. J., Zheng, Z., Zedenius, J., Komminoth, P., Frisk, T., *et al.* Somatic deletions and mutations in the Cowden disease gene, *PTEN*, in sporadic thyroid tumors. Cancer Res., *57:* 4710–4713, 1997.
29. Risinger, J. I., Hayes, A. K., Berchuck, A., and Barrett, J. C. PTEN/MMAC1 mutations in endometrial cancers. Cancer Res., *57:* 4736–4738, 1997.
30. Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., *et al.* Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res., *57:* 3935–3940, 1997.
31. Rhei, E., Kang, L., Bogomolniy, F., Federici, M. G., Borgen, P. I., and Boyd, J. Mutation analysis of the putative tumor suppressor gene *PTEN/MMAC1* in primary breast carcinomas. Cancer Res., *57:* 3657–3659, 1997.
32. Cairns, P., Evron, E., Okami, K., Halachmi, N., Esteller, M., Herman, J. G., *et al.* Point mutation and homozygous deletion of PTEN/MMAC1 in primary bladder cancers. Oncogene, *16:* 3215–3218, 1998.
33. Henderson, Y. C., Wang, E., and Clayman, G. L. Genotypic analysis of tumor suppressor genes *PTEN/MMAC1* and *p53* in head and neck squamous cell carcinomas. Laryngoscope, *108:* 1553–1556, 1998.
34. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., *et al.* Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. Proc. Natl. Acad. Sci. USA, *95:* 5246–5250, 1998.
35. Maxwell, G. L., Risinger, J. I., Gumbs, C., Shaw, H., Bentley, R. C., Barrett, J. C., *et al.* Mutation of the *PTEN* tumor suppressor gene in endometrial hyperplasias. Cancer Res., *58:* 2500–2503, 1998.
36. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1997. CA Cancer J. Clin., *47:* 5–27, 1997.
37. Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J., and Spurr, N. K. Loss of the chromosomal region 10q23–25 in prostate cancer. Cancer Res., *55:* 4800–4803, 1995.
38. Komiya, A., Suzuki, H., Ueda, T., Yatani, R., Emi, M., Ito, H., *et al.* Allelic losses at loci on chromosome 10 are associated with metastasis and progression of human prostate cancer. Genes Chromosomes Cancer, *17:* 245–253, 1996.
39. Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., *et al.* MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res., *57:* 5221–5225, 1997.
40. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., *et al.* Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res., *57:* 4997–5000, 1997.

41. Wang, S. I., Parsons, R., and Ittmann, M. Homozygous deletion of the *PTEN* tumor suppressor gene in a subset of prostate adenocarcinomas. Clin. Cancer Res., *4:* 811–815, 1998.
42. Pesche, S., Latil, A., Muzeau, F., Cussenot, O., Fournier, G., Longy, M., *et al.* PTEN/MMAC1/TEP1 involvement in primary prostate cancers. Oncogene, *16:* 2879–2883, 1998.
43. Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., *et al.* Interfocal heterogeneity of *PTEN/MMAC1* gene alterations in multiple metastatic prostate cancer tissues. Cancer Res., *58:* 204–209, 1998.
44. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res., *58:* 2720–2723, 1998.
45. Gray, I. C., Stewart, L. M., Phillips, S. M., Hamilton, J. A., Gray, N. E., Watson, G. J., *et al.* Mutation and expression analysis of the putative prostate tumour-suppressor gene *PTEN*. Br. J. Cancer, *78:* 1296–1300, 1998.
46. Loda, M., Capodieci, P., Mishra, R., Yao, H., Corless, C., Grigioni, W., *et al.* Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. Am. J. Pathol., *149:* 1553–1564, 1996.
47. Magi-Galluzzi, C., Mishra, R., Fiorentino, M., Montironi, R., Yao, H., Capodieci, P., *et al.* Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. Lab. Invest., *76:* 37–51, 1997.
48. Magi-Galluzzi, C., Montironi, R., Cangi, M. G., Wishnow, K., and Loda, M. Mitogen-activated protein kinases and apoptosis in PIN. Virchows Arch., *432:* 407–413, 1998.
49. Magi-Galluzzi, C., Xu, X., Hlatky, L., Hahnfeldt, P., Kaplan, I., Hsiao, P., *et al.* Heterogeneity of androgen receptor content in advanced prostate cancer. Mod. Pathol., *10:* 839–845, 1997.
50. Fleming, I. D., Cooper, J. S., Henson, D. E., Hutter, R. V. P., Kennedy, B. J., Murphy, G. P., O'Sullivan, B., Sobin, Z. H., and Yarbro, J. W. (eds). American Joint Committee on Cancer. Manual for Staging of Cancer, 5th ed., pp. 220–224. Philadelphia: Lippincott, 1997.
51. Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W. G., Jr. Tumour suppression by the human von Hippel-Lindau gene product. Nat. Med., *1:* 822–826, 1995.
52. Harlow, E., and Lane, D. Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
53. Bostwick, D. Prospective origins of prostate carcinoma. Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia. Cancer (Phila.), *78:* 330–336, 1996.
54. Sano, T., Lin, H., Chen, X., Langford, L. A., Koul, D., Bondy, M. L., *et al.* Differential expression of MMAC/PTEN in glioblastoma multiforme: relationship to localization and prognosis. Cancer Res., *59:* 1820–1824, 1999.
55. Gleason, D. F. Histologic grading of prostate cancer: a perspective. Hum. Pathol., *23:* 273–279, 1992.
56. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., *et al.* High cancer susceptibility and embryonic lethality associated with mutation of the *PTEN* tumor suppressor gene in mice. Curr. Biol., *8:* 1169–1178, 1998.
57. Nefsky, B., and Beach, D. Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. EMBO J., *15:* 1301–1312, 1996.
58. Davies, M. A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., *et al.* Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. Cancer Res., *58:* 5285–5290, 1998.
59. Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G., and Stokoe, D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr. Biol., *8:* 1195–1198, 1998.